UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR II

ÓXIDO NÍTRICO Y ÓXIDO NÍTRICO SINTASAS EN LA APOPTOSIS DE LAS CÉLULAS CROMAFINES BOVINAS

REGULACIÓN Y MECANISMOS DE SEÑALIZACIÓN

TESIS DOCTORAL DE:

ROCÍO PÉREZ RODRÍGUEZ

DIRIGIDA POR:

MARÍA JESÚS OSET GASQUE

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de la Facultad de Farmacia de la Universidad Complutense de Madrid,
bajo la dirección de la Doctora **María Jesús Oset Gasque**

Madrid, 2013

VºBº DIRECTORA DE TESIS
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CERTIFICA

Que Dª ROCÍO PÉREZ RODRÍGUEZ, Licenciada en Bioquímica por la Universidad Complutense de Madrid, y BSc in Biological Sciences por la University of Strathclyde, ha realizado bajo mi dirección el proyecto correspondiente a su Tesis Doctoral "Óxido Nítrico y Óxido Nítrico Sintasas en la apoptosis de las células cromafines bovinas; Regulación y mecanismos de señalización", y que reúne los requisitos necesarios para su presentación como Tesis Doctoral en el Departamento de Bioquímica y Biología Molecular II de la Universidad Complutense de Madrid.

LA DIRECTORA,

Fdo:
Dª María Jesús Oset Gasque

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La presente tesis se presenta en formato publicaciones, con una colección de tres artículos, dos de ellos ya publicados en revistas internacionales, y el último pendiente de publicación. Además se presenta un anexo con tres artículos directamente relacionados con el trabajo de tesis en los que la doctoranda ha participado como autora. El trabajo aquí presentado ha sido realizado con cargo a los siguientes proyectos de investigación:

BFI2003-03886, Ministerio de Ciencia y Tecnología, 2004-2006. "*Las células cromafines como modelo de estudio de la neurodegeneración y neuroprotección*".

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"Los hombres deben saber que es del cerebro, y sólo del cerebro, de donde surgen nuestros placeres, alegrías, risas y bromas, así como nuestras penas, dolores, tristezas y lágrimas. Concretamente, a través de él, pensamos, vemos, oímos y distinguimos lo feo de lo hermoso, lo malo de lo bueno, lo agradable de lo desagradable...Es lo mismo que nos vuelve locos o delirantes, nos inspira miedo o pavor, ya sea de día o de noche (...) Estas cosas que sufrimos proceden todas del cerebro, cuando no está sano (...) Sin embargo cuando el cerebro está tranquilo, un hombre puede pensar correctamente."

Atribuido a Hipócrates, siglo V a. de C.

"Y dónde está el final, lo descubrirás cuando llegues a él."
Balada del Viejo de Leningrado

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A mi Javi, por su paciencia, y por querer estar siempre ahí.

I. Abreviaturas

•OH: Radical hidroxilo
1400W: la N-(3-(aminometil)bencil)acetamidina
3-Br-7-NI: 3-bromo-7-nitroindazol
α1-AR: receptor a-adrenérgico (a-Adrenergic Receptor)
ACH: acetilcolina
ADN: Ácido desoxirribonucleico
Akt /PKB: Proteinquinasa B/Akt
AMPA: Ácido α-amino-3-hidroxi-5-metil-4-isoxazolpropiónico (α-Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid)
Arg: Arginina
ARN: Ácido ribonucleico
ARNm: Ácido ribonucleico mensajero
ATP: Adenosina trifosfato
B2R: receptor B2 de bradiquinina (bradykinin B2 receptor)
Bad: Bcl-xL/Bcl-2 associated death promoter; Bcl-2 antagonist of cell death
Bax: Bcl-2 associated X protein
Bcl-2: B-cell lymphoma/leukemia-2
Bcl-xL: Variante de ayuste alternativo larga de Bcl-x (Bcl-x large)
BF: Prosencéfalo basal
BH4: Tetrahidrobiopterina ((6R)-5,6,7,8-tetrahydro-L-biopterin).
Ca2+: Catión calcio
CaM: Calmodulina.
CaMK: Proteinquinasa dependiente de calcio-calmodulina (Calcium-Calmodulin dependent kinase)
CAPON: proteína ligando del PDZ carboxilo-terminal de nNOS (protein carboxy-terminal PDZ ligand of nNOS)
CAT: transportador de aminoácidos catiónicos (cationic amino acid transporter)
Cav: caveolina-1/-3
CbCx: Corteza cerebelar
CD95 (Fas; APO-1): Receptor de muerte CD95 (Cell Death 95)
CgG: Giro cingulado
cGMP: Guanosina monofosfato 3', 5'-cíclico (ciclic Guanosine-3'-5'-Monophospate)
cit c: Citocromo c
Cl: Claustro
cNOS: NOS constitutiva; Isoforma constitutiva de la óxido nítrico sintasa (constitutive Nitric Oxide
co3-: radical carbonato
CREB: cyclic-AMP-responsive-element- binding protein
c-terminal: carboxilo terminal
Cys (C): Cisteína (Cysteine)
D-AP5: D(-)-2-amino-5-phosphonopentanoic acid
deoxy-Hb: hemoglogina desoxigenada
deoxy-Mb: mioglobina desoxigenada
DETA-NO: Diethylenetriamine NONOate
DLP: depresión a largo plazo, LTD, long term depression
DT: Tálamo dorsal
EBP50: fosfoproteína-50 de unión ezrina/radixina/moesina (ezrin/radixin/moesin-binding phosphoprotein-50)
EGF: Factor de crecimiento epidérmico (Epidermal Growth Factor)
EM: esclerosis múltiple
eNOS (NOS-3, NOS-III): NOS endotelial, isoforma endotelial de la óxido nítrico sintasa (endothelial Nitric Oxide Synthase)
ER: receptor de estrógenos
ERK: Proteinquinasa de regulación extracelular (Extracellular signal-Regulated Kinase)
FAD: Dinucleótido de flavina adenina (Flavine Adenine Dinucleotide)
Fe+2: ión ferroso
Fe+3: ión férrico
FGF-2: factor de crecimiento de fibroblastos
FL: Lóbulo frontal (Frontal Lobe)
FMN: Mononucleótido de flavina (Flavine mononucleotide).
GC: glucocorticoide
GC: Guanilato ciclasa (guanylate cyclase)
Gly (G): Glicina (Glycine)
GR: Glucocorticoid receptor
GSH: Glutatión
GTP: Guanosina 5'-trifosfato
H2O2: peróxido de hidrógeno
HiF: Hipocampo
Hsp70: Proteína de choque térmico 70 (Heat-shock protein 70)
Hsp90: Proteína de choque térmico 90 (Heat-shock protein 90)
Hy: Hipotálamo
IFN-γ: Interferón γ
IGF-1: Factor de crecimiento insulínico tipo 1 (Insulin-like Growth Factor-1)
iGluR = ionotropic glutamate receptor
IκBα: Inhibidor de NF-κB α (Inhibitor of NF-κB α)
IL-1β: Interleuquina 1β
iNOS (NOS-2, NOS-II): NOS inducible; Isoforma inducible de la óxido nítrico sintasa (inducible Nitric Oxide Synthase)
INS: Ínsula
JAK: Janus Kinase
JNK (SAPK): Proteinquinasa N-terminal de Jun (Jun quinasa) (c-Jun N-terminal Kinase)
KA: kainic acid
L-Arg: L-Arginina
L-NMA: N-metil-L-arginina
L-NNA: N-nitro-L-arginina
L-NPA: N-propil-L-arginina
LPS: Lipopolisacárido (Lipopolysaccharide).
L-SMTC: S-metil-L-tiocitrulina
MAPK: Proteinquinasa activada por mitógenos (Mitogen-Activated Protein Kinase)
MCPG: alpha-methyl-4-carboxyphenylglycine
MES: Mesencéfalo
mGluR = metabotropic glutamate receptor
mPTP: Poro de permeabilidad transitoria mitocondrial (mitochondrial Permeability Transition Pore)
mtNOS: NOS mitocondrial; Isoforma mitocondrial de la óxido nítrico sintasa (mitochondrial Nitric
MY: Mielencéfalo
NADH: Nicotinamida adenina dinucleótido reducido
NADP+: Fosfato de nicotinamida adenina dinucleótido oxidado (Nicotinamide Adenine Dinucleotide Phosphate).
NADPH: Fosfato de nicotinamida adenina dinucleótido reducido (Nicotinamide Adenine
NANC: (Neurotransmisión) No-adrenérgica no-colinérgica
NBQX: 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f) quinoxaline
NF-κB: Factor nuclear κB (Factor de transcripción) (Nuclear Factor-κB).
NGF: Factor de crecimiento nervioso (Nerve Growth Factor)
NHA: N-hidroxiarginina (Nω-hydroxy-L-arginine).
NMDA: N-Metil-D-Aspartato
NMDAR: Receptor de NMDA (NMDA Receptor)
nNOS (NOS-1, NOS-I): NOS neuronal; Isoforma neuronal de la óxido nítrico sintasa (neuronal Nitric Oxide Synthase)
NO-: Ión nitrosilo
NO: Óxido nítrico (Monóxido de nitrógeno) (Nitric Oxide).
NO+: nitrosonio
NO_2^-: Dióxido de dinitrógeno
NO_3^-: Anión nitrito
NO^-: Anión nitrato
NOS: Óxido nítrico sintasa (Nitric Oxide Synthase)
NOSIP: proteína que interacciona con las NOS (Nitric Oxide Synthase-Interacting Protein)
NOx : óxidos de nitrógeno
N-terminal: amino terminal
o_2^-: oxígeno molecular
O^-: Anión superóxido
OL: Lóbulo occipital
PDZ: Dominios PDZ (PSD-95 Discs large/ZO-1homology)
PFK-M: fosfofructoquinasa M

PHG: giro parahipocampal
PI3-K : Fosfatidilinositol 3-quinasa (Phosphatidylinositol 3-Kinase)
PIN: Proteína inhibidora de la NOS (Protein Inhibitor of NOS)
PKA: Proteína quinasa dependiente de AMPc (cAMP-dependent Protein Kinase)
PKC: Proteína quinasa dependiente de calcio/fosfolípidos (Protein Kinase C)
PKG: Proteinquinasa dependiente de GMPc (cGMP-dependent Protein Kinase)
PLP: potenciación a largo plazo, LTP long term potentiation
PNMT: feniletanolamina-N metiltransferasa
PSD-95: Proteína 95 de la densidad post-sináptica (Post-Synaptic Density protein 95)
PTg: Tegmento pontino
radicales alquilo (R·), alcoxi (RO·) y alquilperoxi (ROO·)
radicales lipídicos alcoxi (LO·) o alquilperoxi (LOO·).
RG: receptores acoplados a proteínas G
RNS: Especies reactivas de nitrógeno (Reactive Nitrogen Species)
ROS: Especies reactivas de oxígeno (Reactive Oxygen Species)
RyR: Receptor de rianodina (Ryanodine Receptor)
sGC: Guanilato ciclasa soluble (soluble guanylate cyclase)
SNC: Sistema nervioso central
SNP: Sistema nervioso periférico
SOD: Superóxido dismutasa
SR: retículo sarcoplásmico (sarcoplasmic reticulum)
STAT: Signal transducer and activator of transcription
Str: Estriado
TGF-β: Factor de crecimiento transformante-β (Transforming Growth Factor-β)
TL: Lóbulo temporal
TNF-α: Factor de necrosis tumoral-α (Tumor Necrosis Factor).
trans-ACPD: Ácido trans-1-aminociclopentano-1,3-dicarboxílico (trans-1-amino-cyclopentane -1,3-dicarboxylic acid)
VEGF: Factor de crecimiento del endotelio vascular (Vascular Endothelial Growth Factor)
VT: tálamo ventral
XOR: xantina oxido reductasa
α1-Syn: α1-sintrofina (α1-syntrofina)
β-AR: receptor β-adrenérgico (β-Adrenergic Receptor)

II. Índice de contenidos

III. Summary

"Nitric Oxide and Nitric Oxide Synthases in chromaffin cell apoptosis; regulation and signalling mechanisms".

Nitric oxide (NO) is a cellular messenger playing very important roles in physiological processes including regulation of vascular tone, neuronal transmission, and modulation of immunological and inflammatory reactions (Madhusoodanan & Murad 2007). Almost all mammal cells produce endogenous NO, synthesized from L-arginine by three isoforms of NO synthase (NOS) (Bogdan 2001). In the nervous system, the induction of a high output system for NO, in response to cytokines (Liu et al. 2002) or a massive production of glutamate (Nakamura et al. 2007), can result in neurotoxicity and neurodegeneration, mediated by apoptosis. NO plays a dual role on regulation of apoptosis, both pro- and anti-apoptotic. Chromaffin cells are a very useful model of neurosecretary cells (Garcia 2002). Previous studies from our group stated that in these cells NO could mediate cell death, and assessed the presence of the neuronal isoform of NOS (nNOS), as well as cytokine and glutamate receptors (González et al. 1998; Bunn et al. 2012). Therefore, in this work we tried to determine the involvement of exogenous and endogenous NO on apoptotic cell death.

In the first part of this work, we aimed to establish the possible involvement of mitochondria in the apoptotic events triggered by exogenous nitric oxide (NO) in chromaffin cells. Using bovine chromaffin cells in primary culture and several NO donors (SNP, SNAP, and GSNO) at apoptotic concentrations (50 μM–1 mM), we have shown that NO induces a time-dependent decrease in the mitochondrial transmembrane potential (Ψm), which correlates to the appearance of hypodiploid cells. Disruption in Ψm is followed by cytochrome c release to the cytosol, which in turn precedes caspase 3 activation. The Bcl-2 protein family participates in this mechanism. NO donors downregulate the expression of anti-apoptotic members of the family, such as Bcl-2 and Bcl-XL, and increase the expression of pro-apoptotic members, Bax and Bcl-Xs, inductors of cytochrome c release to cytosol. Different cell signaling pathways seem to regulate Bax induction and Bcl-2 inhibition because decreased Bcl-2 levels are detected later than enhanced Bax expression. The tumor suppressor protein p53 is also upregulated in a very early phase (30 min) of the NO-induced apoptosis and may be responsible for the further induction of Bax expression. Finally, the translocation of NF-κB to the nucleus seems to be another early event in NO-induced apoptosis and it may be in-

volved in the regulation of p53 expression. All together, these results support the participation of mitochondrial mechanisms in NO-induced apoptosis in chromaffin cells.

Glutamate is the main excitatory neurotransmitter in the nervous system, having important functions in memory and neuronal plasticity, but it is also involved in excitotoxicity and neuronal cell death (Bliss & Collingridge 1993; Kostandy 2012). Several lines of evidences shows an important functional relationship between NO and glutamate (Nakamura et al. 2007). Therefore, in this work we want to characterize the expression of different glutamate receptors and to know if glutamate regulates the catecholamine (CA) secretion. We proved that chromaffin cells express various ionotropic (NMDA, AMPA, KA) and metabotropic (mGluR1, mGluR5) glutamate receptors, exerting different effects on the regulation of CA secretion. Results obtained in the presence of specific agonists and antagonists of these glutamate receptors demonstrate the involvement of glutamate in the regulation of CA secretion, via NO/cGMP.

Then, in the second and third part of this work, we tried to assess the effect of endogenously generated NO on chromaffin cell apoptosis, both under basal conditions and stimulated with cytokines and glutamate. As the endogenous source of NO in mammalian cells seems to be the family of NOS, we characterized the NOS isoforms present in chromaffin cells. We found out that these cells express nNOS under basal conditions, and iNOS under cytokine induction. Therefore, we tried to assess the specific regulation of nNOS and iNOS, and their particular participation in cell death induced by cytokines and glutamate. Our results show that cytokines, glutamate and glutamate agonists are able to induce cell death and apoptosis in bovine chromaffin cells, in parallel to an increase in nitrite production.

In order to assess whether or not NOS were mediating these effects, cells were treated with specific NOS inhibitors. The following inhibitors were used: the specific iNOS inhibitor W-1400, and some anyhow specific nNOS inhibitors: N-ω-propyl-L-arginine (N-PLA), 3-Bromo-7-nitroindazol (7-NI), L-methyl thiocitrulline (thiocitrulline), and N-methyl L-arginine (L-NMA). Under basal

conditions, iNOS inhibitors did not have any effect on apoptosis nor nitrite production, whereas nNOS inhibitors induced apoptosis, indicating a neuroprotective effect of constitutive nNOS-generated NO. Under cytokine (IFN-γ) stimulation, cell death was abolished by W-1400, but only partially by nNOS inhibitors (N-PLA, 7-NI, thiocitrulline and L-NMA), indicating that iNOS is the main isoform participating in cytokine-induced chromaffin cell death. In contrast, glutamate-induced cell death and apoptosis was strongly reverted by nNOS inhibitors and weakly by iNOS inhibitors, thus indicating the nNOS involvement, but not iNOS, in glutamate-mediated apoptosis.

We also wanted to clear out whether these compounds had any effects on NOS expression. We found that lipopolysaccharide (LPS) and IFNγ increased iNOS mRNA levels and protein expression, with no effect on nNOS. On the other hand, dexamethasone increased basal nNOS mRNA levels and protein expression but decreased LPS + IFNγ-induced iNOS expression. On the contrary, glutamate and glutamate agonists specifically activated nNOS mRNA levels and protein expression, but had no effects on iNOS.

So far, we had clear evidence on the role of glutamate and cytokines on apoptosis of chromaffin cells, but its specific mechanisms were unclear. Therefore, we pointed at some of the possible involved pathways. NF-κB is a transcriptional factor that, when translocated to the nucleus, stimulates iNOS expression. We found that IFNγ and LPS, as well as nNOS inhibitors, induced NF-κB translocation to the nucleus. This was indeed a specific process, since SN50, which inhibits NF-κB translocation, decreased iNOS expression. On the other hand, glutamate could not activate NF-κB. In addition to this mechanism of action, IFNγ and LPS induced 847Ser nNOS phosphorylation, inhibiting nNOS activity. Both processes, nNOS phosphorylation and iNOS expression induced by LPS + IFNγ, are regulated by the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, as IFNγ increases 727STAT-3 phosphorylation and specific inhibitors of JAK/STAT pathway, such as AG490, inhibited both processes. Also, our results seem to suggest the participation of PKG, PKA, PKC and MAPKs pathways in glutamate-mediated nNOS activation in chromaffin cells and point out the involvement of both PKA and PKC

signaling pathways in the apoptotic effect of glutamate.

In short, at the view of our results and the evidences on the literature about endogenous NO and apoptosis, we propose a model to explain the effects of NO, both endogenous and cytokines/glutamate stimulated on bovine chromaffin cells. In basal conditions, chromaffin cells express only nNOS, having nNOS inhibitors an apoptotic effect. Therefore, in basal conditions nNOS is anti-apoptotic and cytoprotective, promoting cell survival. nNOS produces physiological NO concentrations that would arrest NF-κB activation. Cytokines promote nNOS phosphorylation, diminishing NO basal levels, and thus allowing NF-κB translocation to the nucleus, and DNA binding. This would in turn activate iNOS gene expression, increasing iNOS protein expression and producing great amounts of NO, involved in apoptosis. NO would produce a feedback inhibition of NF-κB activation. Glutamate, possibly through NMDA- and non NMDA-type iGluRs and mGluRs, would activate nNOS, producing NO enough to stimulate apoptosis and inhibit NF-κB activation. nNOS inhibitors would counteract these glutamate-induced NO levels, improving cell survival, but yet not activating NF-κB since NO concentration remains high. However, NO concentrations much lower than basal levels –obtained through enzyme inhibitors or phosphorylation- would block NF-κB inhibition, contributing to iNOS activation and apoptosis. In the same way, very high levels of glutamate-generated NO, fast effect, or cytokine generated NO, slow effect, would restrain induced apoptosis. All together, these results stand for chromaffin cells as a good model for study of neurodegeneration and neuroprotection, in which both glutamate, cytokines and NO are strongly involved.

IV. Introducción

Explicación Introducción

La introducción de esta tesis se ha diseñado para poder leerse de dos maneras. La vía larga, tal y como se leería un capítulo de libro normal. La vía corta, mediante los resúmenes que aparecen en azul en los márgenes de cada página, y las figuras y los pies de figuras.

Además, se ha echo hincapié en la bioquímica del óxido nítrico, aunque este tema no se trata directamente en la tesis doctoral. El óxido nítrico es una molécula sencilla, pero sus implicaciones fisiológicas son numerosísimas. La única manera de poder comprender todas las reacciones en las que está implicado es mediante el estudio de su química. Por mi formación, este tema siempre me ha parecido muy interesante, pero para el desarrollo de mi tesis ha sido fundamental. La mayoría de los estudios no prestan atención a estos detalles, y por ello he decido incluirlo en esta revisión.

Espero que lo disfruten.

A. Óxido nítrico

"Cuando Robert Furchgott, Louis Ignarro y Ferid Murad descubrieron, de manera independiente, que un gas de vida muy breve, el NO, se producía endógenamente y actuaba como molécula señalizadora entre células – fue inesperado y único. Inició un nuevo capítulo en la investigación biomédica y se abrieron nuevos horizontes.

Fue Robert Furchgott quién abrió el camino en 1980. Durante los años 70, se había observado que la capa celular interna de los vasos sanguíneos, el endotelio, no sólo tenía propiedades pasivas y protectoras. Furchgott demostró, de manera inesperada, que la contracción y la relajación de los vasos sanguíneos dependían de la presencia del endotelio. En un brillante experimento – el llamado experimento sándwich- hizo un descubrimiento crucial que sentó las bases para futuras investigaciones científicas. En este estudio se observaron las respuestas de distintas secciones de la aorta. Una sección tenía la capa endotelial intacta, mientras que en la otra se había eliminado. En ausencia de endotelio, Furchgott registró una contracción que respondía a la estimulación. La sección con endotelio se preparó de manera tal que no se pudieran dar ni la contracción ni la relajación. Cuando juntó las dos secciones en un modelo "sándwich", observó que esa misma estimulación ya no producía una contracción, sino una relajación. Furchgott concluyó que en el endotelio se producía una sustancia desconocida, un factor, que se transportaba hasta la sección de la aorta sin endotelio y que eso provocaba la relajación.

Fue gran descubrimiento. Dio el pistoletazo de salida de una búsqueda de la identidad de este factor endotelial. Una búsqueda que duró seis años. Se lanzaron diferentes hipótesis. Una de ellas señalaba que debía haber implicados compuestos nitrogenados. En este campo de la investigación se movía Ferid Murad. Sabía que la nitroglicerina activaba una enzima en las células musculares aórticas, la guanilato cliclasa (GC), que aumentaba el cGMP (guanosina monofosfato 3', 5'-cíclica) y provocaba relajación. En este momento Ferid Murad hizo una pregunta importante: ¿Actuaba la nitroglicerina liberando NO? Probó esta hipótesis burbujeando NO gaseoso a través de una preparación de tejido con GC. La producción de cGMP aumentó. Se acababa de descubrir una nueva forma en que las drogas activaban la función de una enzima. El principio de

acción de la nitroglicerina, desconocido hasta ahora a pesar de los más de 100 años de exitosos tratamientos de la angina de pecho, se había descubierto. Estos experimentos llevados a cabo por Ferid Murad, unos años después del descubrimiento de Furchgott del factor endotelial, crearon un conocimiento nuevo, que posteriormente se convertiría en la clave de la identificación del factor endotelial.

Fue en este camino en el que el tercer premiado, Louis Ignarro, conducía sus actividades y experiencia científica. Inspirado por los descubrimientos de Murad, también observó que el NO relajaba la vasculatura. De manera simultánea e independiente a Robert Furchgott, también añadió, durante la primera mitad de los años 80, nuevos descubrimientos sobre este factor. Su identidad era cada vez más clara. La búsqueda del desconocido factor endotelial de Furchgott acabó en un congreso científico en la Clínica Mayo, en Rochester, Minnesota, en el verano de 1986. En el congreso Furchgott concluyó, basándose en varios hallazgos, que el factor era idéntico al NO. Ignarro apoyó esta teoría en el mismo congreso, y fue más allá, con un interesante experimento. Utilizó el análisis de espectro, lo que significa que cada sustancia emite un espectro único y específico. Encontró espectros idénticos cuando la hemoglobina reactiva reaccionaba con el factor endotelial, y cuando reaccionaba con el NO, y concluyó que el factor era el NO.

La búsqueda había acabado. El misterio del factor endotelial se había resuelto. Un gas endógeno de vida breve, con capacidad para actuar como una molécula señalizadora entre las células del cuerpo. Era un fenómeno nuevo. El descubrimiento explicaba el mecanismo de acción de la nitroglicerina al tratar la tensión arterial y la angina, enfermedad que padeció Alfred Nobel…El descubrimiento de que el factor endotelial era el NO también abrió la puerta a nuevas terapias en la medicina clínica, mejoró la capacidad de diagnóstico de enfermedades inflamatorias graves y abrió nuevas posibilidades para el desarrollo de medicamentos. La investigación en el campo del NO desde 1986 hasta nuestros días es enorme. Profesores Robert Furchgott, Louis Ignarro y Ferid Murad: Sus descubrimientos sobre el NO como molécula señalizadora en el sistema cardiovascular, no sólo han explicado los mecanismos de acción de un antiguo e importante grupo de medicamentos, los nitro vasodilatadores, sino que además han abierto nuevos caminos para el tratamiento y diagnóstico de muchas enfermedades. Sus descubrimientos han elevado la investigación médica a una nueva era. (…)"

1. Una visión general

El óxido nítrico o monóxido de nitrógeno (NO) es una de las moléculas más pequeñas y ubicuas que se conocen y un importante metabolito en las células de los mamíferos (Moncada et al. 1991). Su importancia se constata al observar que, hasta la fecha, no se han descrito células nucleadas de mamíferos que no sean capaz de sintetizar NO (Bogdan 2001).

El NO es una de las moléculas más pequeñas y ubicuas que se conocen y un importante metabolito en las células de los mamíferos. Hasta la fecha, no se ha descrito ninguna célula nucleada de mamíferos que no sea capaz de sintetizar NO.

Físicamente el NO es un gas incoloro, pequeño (30 Da) y de carga neutra, lo que le permite difundir velozmente (50 µm/s) a través de las membranas biológicas de la mayor parte de los tejidos (Aktan 2004), pero no a través de la vasculatura, ya que reacciona inmediatamente con la oxihemoglobina (Beckman & Koppenol 1996). Así, es capaz de pasar de una célula a otra a una velocidad superior a la que tienen lugar las reacciones intracelulares, por lo que la concentración de NO en el interior de una célula está determinada por el número de células productoras de NO que la rodean (Murphy 1999). El NO es un radical libre con un electrón desapareado, que existe en las células por un breve periodo de tiempo (de 6 a 10 segundos) antes de convertirse en nitrato (NO_3^-) o nitrito (NO_2^-) (Tayfun & Oglesby 2001). En solución acuosa tiene una baja solubilidad: 1,95 mM/atm a 25ºC (Trostchansky et al. 2010).

La gran variedad de reacciones en las que está implicado el NO se basan en, primero, el amplio número de células en las que se sintetiza. Segundo, su naturaliza hidrofóbica, que le permite difundir fácilmente a través de las membranas, y no restringe su actividad a su sitio de formación. Tercero, los diferentes estados de oxidación en los que puede encontrarse el nitrógeno. En la forma reducida se forma nitrosilo (NO^-), y en la forma oxidada se obtiene ión nitrosonio (NO^+) (Mariotto et al. 2004). Todo ello le confiere una gran capacidad de interacción, ya que reacciona con moléculas inorgánicas (oxígeno (O_2), superóxido ($O_2^{-\cdot}$) y metales de transición), grupos prostéticos (hemo), y proteínas (participando en la S-nitrosilación de grupos tioles y nitración de residuos de tiroxina), siendo muchos de estos receptores por si mismos moléculas reguladoras (Bogdan 2001).

Por todas las peculiaridades antes descritas, se observa que la función que desempeña el NO en el organismo sea compleja, ya que juega innumerables papeles en tejidos y circunstancias completamente diferentes. En los riñones y el sistema cardiovascular, controla el tono vascular, la microcirculación glomerular y la secreción de sodio renal. En las células endoteliales es un agente vasodilatador y un factor de supervivencia. El NO también está íntimamente relacionado con el correcto funcionamiento de la respuesta inmune, controla procesos inflamatorios como artritis, miocarditis, colitis, nefritis y está relacionado con las grandes patologías de nuestro siglo, como diabetes, cáncer y sida (Kone et al. 2003; Krumenacker et al. 2004; Bishop & Anderson 2005).

En el sistema nervioso periférico (SNP), el NO controla la secreción hormonal, la visión, la respiración, etc. En el sistema nervioso central (SNC), juega un papel crítico en diversas funciones: memoria y aprendizaje, secreción de neurotransmisores, ansiedad, plasticidad sináptica, sinaptogénesis, regulación en la expresión de ciertos genes, percepción del dolor y regulación de los ciclos circadianos. Está asociado con la dependencia física a drogas, con el daño neural causado por el etanol y también con procesos neurodegenerativos como las enfermedades de Parkinson, Alzheimer, Huntington, demencia asociada al VIH e isquemia cerebral. Sin embargo, lo que realmente convirtió esta ubicua molécula en el blanco de todas las miradas fue su función como neurotransmisor, ya que aunque cumple algunos de los criterios definidos para este tipo de moléculas (difunde de una neurona a otra, y tiene un receptor hierro hemínico en el sitio activo de la GC), no cumple otros (no se almacena en las vesículas sinápticas, no se libera por exocitosis, y su receptor es la GC y no un receptor de membrana), y por ello su descubrimiento revolucionó el concepto clásico de neurotransmisores (Dawson 1998; Dawson 2004; Goligorsky et al. 2002; Tayfun & Oglesby 2001).

2. Estructura molecular

El NO es un radical libre relativamente estable. Las reacciones químicas en las que participa se basan en su naturaleza como radical libre, o en la habilidad del nitrógeno para cambiar su estado de oxidación.

La molécula de NO está formada por un átomo de nitrógeno y uno de oxígeno. El oxígeno y el nitrógeno tienen una electronegatividad muy similar, lo que confiere a la molécula de NO un momento dipolar muy pequeño, siendo una molécula prácticamente hidrofóbica que difunde muy fácilmente a través de las membranas lipídicas (Mariotto et al. 2004).

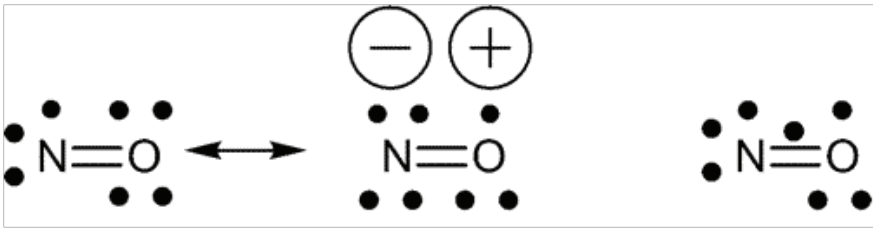


Imagen 1 *La molécula de NO (McCleverty 2004)*

La estructura molecular del NO se entiende en términos de la ocupación de sus orbitales moleculares. Los orbitales atómicos del N (un orbital 2s) y del O (tres orbitales 2p) se combinan para formar ocho orbitales moleculares, cuatro enlazantes (σ2s, 2 π2p, σ2p) y cuatro antienlazantes (σ·2s, 2 π·2p, σ·2p). De este modo, quedan ocho electrones situados en orbitales enlazantes y tres en orbitales antienlazantes, y un electrón desapareado (Mariotto et al. 2004) en el orbital molecular antienlazante p*. Esta disposición de electrones forma una estructura intermedia entre enlace doble y triple, lo que le confiere un orden de enlace de 2,5 (Trostchansky et al. 2010). Esta estructura es consistente con la longitud de enlace observada de 1,150Aº. Los radicales libres se definen como especies

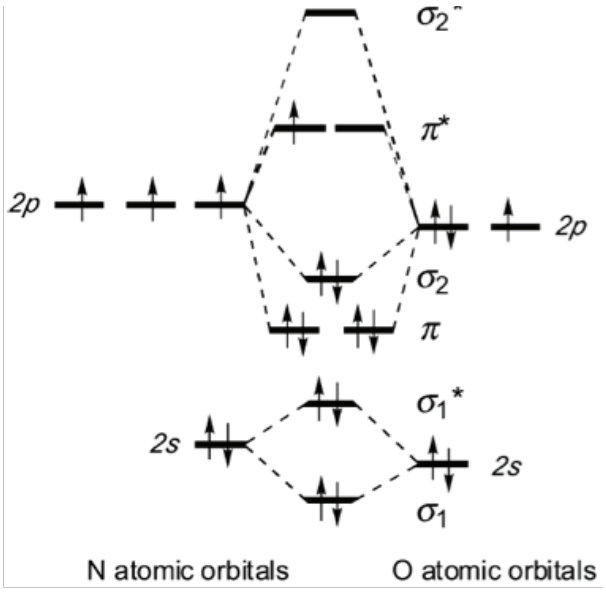


Imagen 2 *Diagrama de orbitales moleculares del NO. (Según McCleverty)*

de existencia independiente que contienen uno o más electrones desapareados ocupando, ellos solos, un orbital atómico o molecular. Estos electrones desapareados confieren a la molécula que es el radical libre reactividad química. Como hemos visto anteriormente, el NO es un radical libre relativamente estable (Bruckdorfer 2005), que reacciona exclusivamente con otras especies paramagnéticas, tales como otros radicales o metales (Trostchansky et al. 2010).

3. Formación

Las óxido nítrico sintasas (NOS) catalizan una oxidación de cinco electrones dependiente de oxígeno, para formar NO y L-citrulina a partir del aminácido L-arginina (L-Arg). Hasta hace poco se pensaba que las NOS eran las únicas responsables de la formación de NO en los mamíferos, pero se ha demostrado que hay una ruta alternativa para la formación de NO. Esta ruta se ha denominado ciclo de nitrato-nitrito-NO, en el que los iones inorgánicos NO_3^- y NO_2^- se vuelven a convertir en NO en sangre y tejidos. La primera etapa, reducción de NO_3^- a NO_2^-, la realizan bacterias comensales, mientras que la conversión de NO_2^- en NO está mediada por enzimas mamíferas (Lundberg et al. 2008; Lundberg & Weitzberg 2010a; Lundberg & Weitzberg 2010b).

Las óxido nítrico sintasas (NOS) catalizan una reacción dependiente de oxígeno, para formar NO y L-citrulina a partir del aminácido L-arginina. Hasta hace poco se pensaba que las NOS eran las únicas responsables de la formación de NO en los mamíferos, pero se ha demostrado que existe una ruta alternativa denominada ciclo de nitrato-nitrito-NO, en el que estos iones inorgánicos se vuelven a convertir en NO en sangre y tejidos.

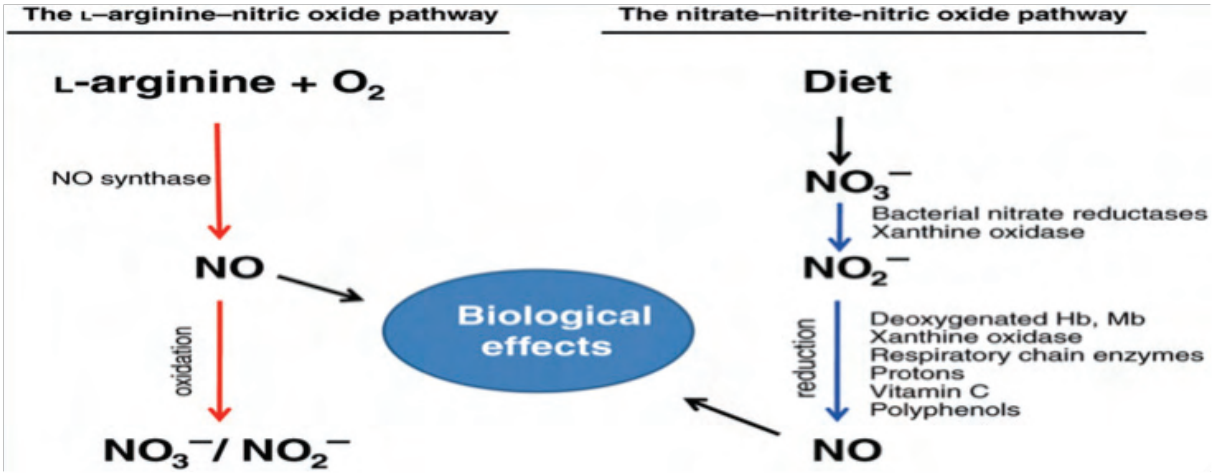


Imagen 3 Rutas de la generación de NO en mamíferos. Existen dos rutas paralelas para la formación de NO en mamíferos. Las NOS catalizan la formación de NO a partir de los sustratos L-Arg y O_2 . El NO se oxida rápidamente para formar nitritos y nitratos, pero estos productos se pueden reciclar para volver a formar NO. La reducción de nitratos a nitritos se produce principalmente por bacterias comensales en la cavidad oral y, en menor medida, por enzimas que se encuentran en los tejidos de mamíferos (xantina oxidasa). Tras la formación del nitrito existen diversas rutas en sangre y tejidos por las que se puede metabolizar los nitritos a NO y a otros óxidos de nitrógeno. La mayor parte de estas rutas se aceleran en condiciones hipóxicas. La reducción de los nitritosrepresenta una alternativa a la síntesis de NO mediada por las NOS. La mayor contribución a la reserva de nitratos se produce a través de la dieta (principalmente verduras de hojas verdes), y abastece la ruta nitrato-nitrito-NO. La ingesta de nitratos se asocia con un potente efecto similar al producido por NO, entre los que destaca la reducción de la presión arterial y la inhibición del consumo de oxígeno. Además, la administración de nitratos o nitritos juega un papel protector en modelos animales de enfermedad cardiovascular (Lundberg et al. 2011).

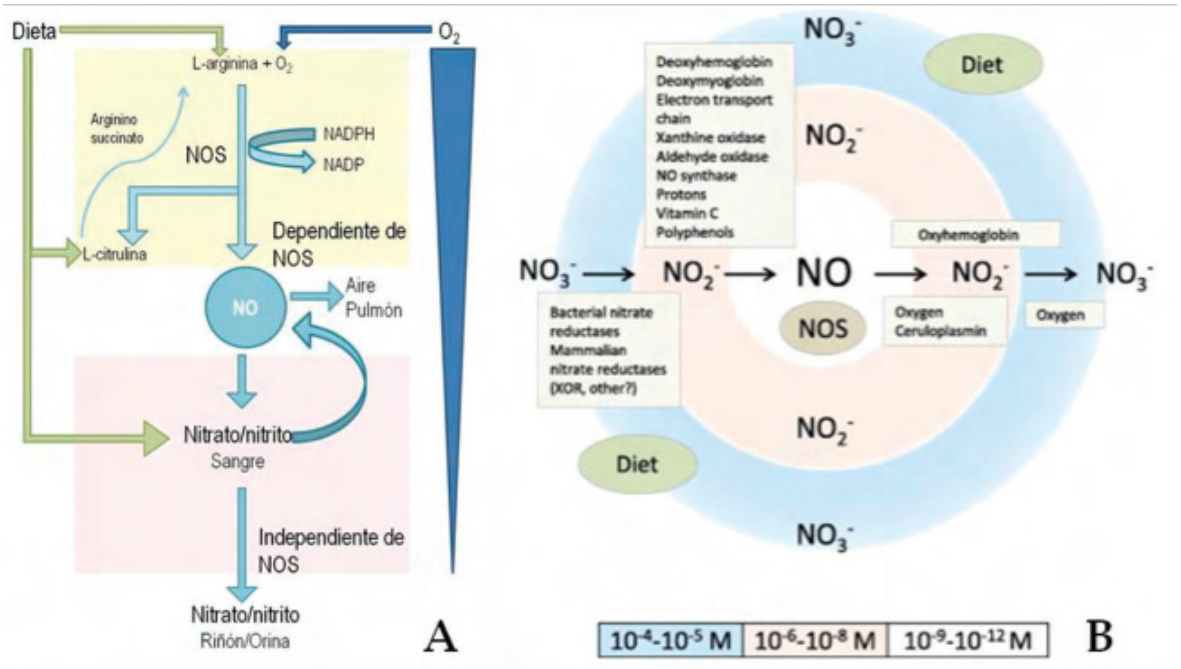


Imagen 4. Síntesis de NO en humanos. A. Dependencia de Óxigeno (adaptación de (Bescós et al. 2012). La ruta dependiente de NOS necesita O2, pero la ruta nitrato-nitrito-NO se activa progresivamente en respuesta a la caída de tensión de O2).B. Concentraciones de NO (Lundberg & Weitzberg 2010b). La vida media del NO en los fluidos biológicos es extremadamente corta, debido a su rápida oxidación a NO2– y NO3–. En la imagen se muestran las concentraciones de NO3–, NO2– y NO en sangre y tejidos presentados.

Por último, mientras que la ruta dependiente de NOS se produce por oxidación en condiciones aeróbicas, la generación de NO por bacterias es una reacción de reducción en condiciones anaeróbicas. Se ha demostrado que a tensiones de oxígeno muy bajas, se puede generar NO en los tejidos de manera independiente de NOS y dependiente de NO_2^-. Así, la formación de NO independiente de NOS se podría ver como un sistema de apoyo complementario para garantizar una concentración de NO suficiente cuando el aporte de O_2 es limitado (Lundberg et al. 2008).

Varias líneas de investigación convergen en la idea de que la vía nitrato-nitrito-NO tiene una función fisiológica, nutricional y terapéutica, y complementa la vía clásica L-Arg-NOS-NO, principalmente en condiciones hipóxicas cuando las isoformas de las NOS, dependientes de oxígeno, no son funcionales (Lundberg et al. 2008). La formación de NO dependiente de NOS se tratará en el capítulo dedicado a las NOS.

4. Translocación y almacenamiento

Tras su síntesis, el NO tiene que difundir o ser transportado lejos de las NOS para ejercer sus funciones biológicas. Las características específicas de la difusión del NO se analizan más adelante, pero hay que tener en cuenta que el NO es una molécula gaseosa que difunde libremente y no se puede almacenar en vesículas. Algunos estudios sugieren que el tiempo de vida del NO en entornos biológicos aeróbicos podría ser demasiado corto para dar lugar a todas las reacciones observadas (Tennyson & Lippard 2011). Sin embargo, el NO tiene dos mecanismos que compensan, en parte, estos problemas.

Tras su síntesis, el NO tiene que difundir o ser transportado lejos de las NOS para poder ejercer sus funciones biológicas. Como el NO es una molécula gaseosa que difunde libremente, no se puede almacenar en vesículas. Sin embargo, tiene dos mecanismos compensatorios: Por un lado, reacciona con moléculas que sirven como vesículas de "almacenamiento" o de suministro de NO. Por otro, las NOS utilizan la síntesis localizada de NO, ubicándose próximas a las dianas moleculares del NO.

Por un lado, el NO puede reaccionar con moléculas que sirvan como vesículas de "almacenamiento" o vehículos de suministro que guarden el NO o lo transporten cuando sea necesario. En los tejidos, estos "almacenes" de NO podrían ser otros óxidos de nitrógeno: por ejemplo, nitratos y nitritos podrían servir como almacenes de NO, ya que siguen reacciones fisiológicas de reciclado en sangre y tejidos para formar NO y otros óxidos de nitrógeno (Lundberg et al. 2008). También podrían ser proteínas S-nitrosiladas, como los S-nitrosotioles, y complejos metal-nitrosilo. Estas moléculas pueden liberar NO por descomposición térmica, en procesos catalizados por metales o iluminación (Tennyson & Lippard 2011).
Por otro lado, las NOS utilizan la síntesis localizada de NO, ubicándose próximas a las dianas moleculares del NO, en microdominios específicos de las células (Govers & Oess 2004).

5. Química del óxido nítrico

La bioquímica del NO es extremadamente compleja y depende de variables tales como el lugar, el momento o la concentración de NO presente o producido en determinadas circunstancias y durante un cierto tiempo. Por otra parte, las reacciones en las que participa el NO se pueden explicar según a) la naturaleza de radical libre de la molécula de NO, y b) la habilidad del nitrógeno para cambiar su estado de oxidación. Además, el estudio de la bioquímica del NO se debe ampliar a todas las especies de nitrógeno relacionadas.

Al contrario de lo que pueda parecer, el NO es poco reactivo con la mayoría de las moléculas biológicas (Reiter 2006). No sigue el prototipo de interacciones proteína-receptor, o de pequeñas moléculas, sino que reacciona formando uniones covalentes. Inicialmente, las investigaciones con NO intentaron evaluar las modificaciones químicas y bioquímicas de sus dianas moleculares, pero según avanzaban los estudios en este campo, se observó que la bioquímica del NO es extremadamente compleja y depende de variables tales como el lugar, el momento o la concentración de NO presente o producido en determinadas circunstancias y durante un cierto tiempo, como veremos más adelante.

Aparte de las anteriores consideraciones, las reacciones en las que participa el NO, gas de carga neutra a pH fisiológico, se pueden explicar según sus características químicas: por una parte la naturaleza de radical libre de la molécula de NO y, por otra, la habilidad del nitrógeno para cambiar su estado de oxidación.

Como radical libre, el NO reacciona exclusivamente con otras especies paramagnéticas, tales como otros radicales o metales. Las velocidades de reacción del NO con radicales son generalmente rápidas, aproximándose a los límites de difusión para algunos, mientras que la reacciones con centros metálicos o con oxígeno unido a centros metálicos ocurren más despacio (Trostchansky et al. 2010).

Según el estado de oxidación del nitrógeno, el NO se puede dar en tres formas: NO•, NO+, NO−.Al reducir el NO, añadiendo un electrón más, se obtiene el NO−, donde el nitrógeno tiene un estado de oxidación +1. Al oxidar el NO eliminando el electrón desapareado, se obtiene NO+, donde el nitrógeno tiene un estado de oxidación +3. (Mariotto et al. 2004). La existencia de estas tres formas redox permite establecer reacciones cruzadas con el oxígeno, dióxido de carbono, peróxido de hidrógeno (H_2O_2), anión superóxido y metales de transición (Gautier-Sauvigné et al. 2005).

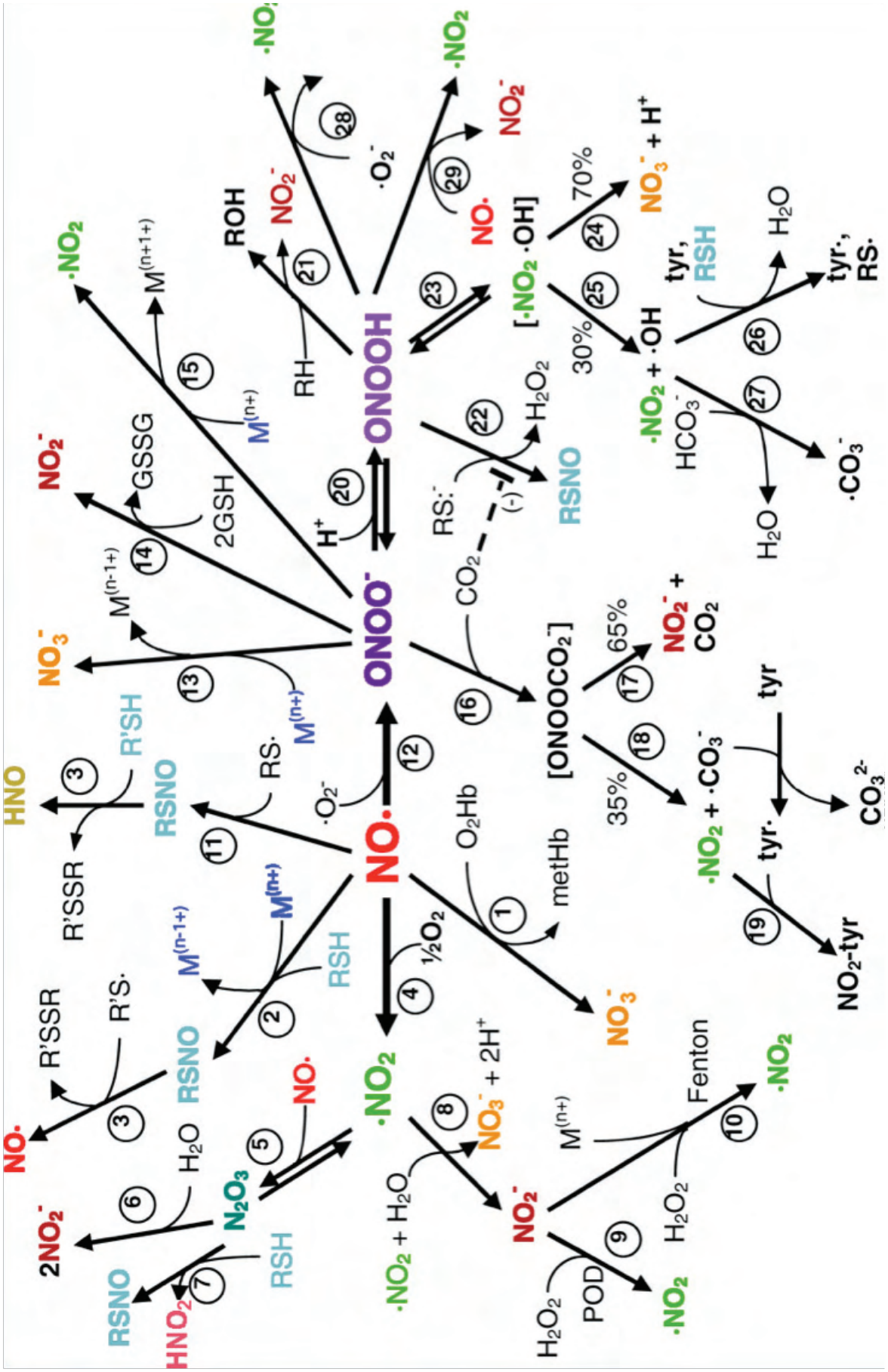
Las principales reacciones inducidas por el NO son:

1. Nitrosación: adición de un grupo nitroso (-NO+) en otro grupo reactivo o fracción molecular, como en los compuestos C-nitroso, S-nitroso y N-nitroso.

2. Nitrosilación: adición de un grupo (-NO) (S-Nitrosilación), que indica modificación postraduccional de proteínas al unirse a un radical NO. La S-nitrosilación modifica la actividad de estas proteínas de manera similar a la fosforilación, en un proceso reversible. De este modo el NO participa en mecanismos regulatorios celulares (Stamler et al. 2001; Korhonen et al. 2005).

3. Nitración: incorporación de un grupo nitro (-NO2).
   En general, y aunque se trate de dos reacciones diferentes, en la bibliografía se tiende a confundir los términos de nitrosación y nitrosilación (d'Ischia et al. 2011; Ricciardolo et al. 2006).

En la siguiente tabla se resumen las reacciones en las que está implicado el NO.


NO·
·NO2
ONOO⁻
ONOOH
[ONOOCO2⁻]
[·NO2 ·OH]
·NO2 + ·OH
·NO2 + ·CO3⁻
NO2⁻
NO3⁻
·O2⁻
H2O2
RSNO
RSH
R'SH
R'SSR
R'S·
RS·
RS:⁻
HNO
2NO2⁻
HNO2
N2O3
O2Hb
metHb
½O2
CO2
HCO3⁻
·CO3⁻
CO3²⁻
NO2-tyr
tyr
tyr·
tyr·, RS·
tyr, RSH
H2O
ROH
RH
GSSG
2GSH
M(n+)
M(n-1+)
M(n+1+)
H+
POD
Fenton
·NO2 + H2O
NO3⁻ + 2H+
·NO2 + ·CO3⁻
NO2⁻ + CO2
NO3⁻ + H+
70%
30%
65%
35%
(-)
1
2
3
4
5
6
7
8
9
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11
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N	Reactivos	Productos	Reacción	Comentarios
1	$NO\cdot + O_2Hb$	$NO_3^- + metHb$	Oxidación de NO	
2	$NO\cdot + RSH, M^{n+}$	$RSNO, \quad M^{n-1+}$	S-nitrosilación de tioles	Catalizada por metales de transición (M)
3	$RSNO + R'S\cdot$	$R'SSR + NO\cdot$ ó HNO	Liberación de NO	RSNO como almacén de NO
4	$NO\cdot + O_2$	$NO_2\cdot$	Oxidación de NO	
5	$NO\cdot + NO_2\cdot$	N_2O_3	Autooxidación de NO y NO2	
6	$N_2O_3 + H_2O$	NO_2^-	Hidrólisis de N2O3	
7	$N_2O_3 + RSH$	$HNO_2 + RSNO$	Nitrosación	
8	$NO_2\cdot + NO_2\cdot$	$NO_2^- + NO_3^- + H^+$	Oxidación De NO2	En solución acuosa
9	$NO_2^- + H_2O_2$, POD	$NO_2\cdot$	Reciclado de NO2	Mediante peroxidasa (POD)
10	$NO_2^- + H_2O_2, M^{n+}$	$NO_2\cdot$	Reciclado de NO2	Catalizada por M, Reacción de Fenton
11	$NO\cdot + RS\cdot$	RSNO	Condensacio de radicales	
12	$NO\cdot + O_2^-\cdot$	$ONOO^-$	Condensación de radicales	
13	$ONOO^-, M^{n+}$	NO_3^-, M^{n-1+}	Oxidación	Catalizada por metales de transición (M)
14	$ONOO^- + GSH$	$NO_2^- + GSSG$	Detoxificación	Mediante selenoenzimas
15	$ONOO^-, M^{n+}$	$NO_2\cdot, M^{n+1+}$	Reducción	Catalizada por M
16	$ONOO^- + CO_2$	$ONOOCO_2$	Condensación	
17	$ONOOCO_2$	$NO_2^- + CO_2$	Descomposición	(35%)
18	$ONOOCO_2$	$NO_2\cdot + \cdot CO_3^-$	Descomposición en radicales	(65%)
19	$NO_2\cdot + \cdot CO_3^- + Tyr$	$NO_2Tyr + CO_3^{2-}$	Nitración	Previa oxidación de Tyr por $\cdot CO_3^-$
20	$ONOO^- + H^+$	ONOOH	Protonación	
21	$ONOOH + RH$	$ROH + NO_2^-$	Oxidación de hidrocarburos alifáticos y aromáticos	
22	$ONOOH + RS\cdot$	RSNO	S-nitrosilación de tioles	Inhibida a concentraciones biológicas de CO_2
23	ONOOH	$[NO_2\cdot \ OH^-]$	Descomposición	Producto intermedio
24	$[NO_2\cdot \ OH^-]$	$NO_3^- + H^+$		70%
25	$[NO_2\cdot \ OH^-]$	$NO_2\cdot + OH^-$		30%
26	$OH^- +$ biomoléculas	Radicales	Oxidación de biomoléculas	
27	$OH^- + HCO_3^-$	$\cdot CO_3^- + H_2O$	Oxidación	
28	$ONOOH + O_2^-\cdot$	$NO_2\cdot$	Descomposición	
29	$ONOOH + NO\cdot$	$NO_2\cdot + NO_2^-$	Descomposición	

Imagen 5 y Tabla 1. Bioquímica del NO y sus derivados. Resumen de las reacciones bioquímicas del NO demostradas empíricamente. Los números en círculos de la imagen superior se refieren a las reacciones y rutas de la tabla inferior (Manukhina et al. 2006).

5.1. Óxidos de nitrógeno

Como se puede observar, el estudio de la química del NO es muy complejo. Ése no es el único problema. Por una parte, se sabe que el NO es muy reactivo e inestable en los entornos biológicos (aeróbicos, en soluciones acuosas, y en presencia de iones metálicos y compuestos orgánicos). En condiciones fisiológicas, la vida media del NO varía de milisegundos hasta minutos. Por todo ello, algunos estudios sugieren que las concentraciones intravasculares de NO podrían ser demasiado bajas para proporcionar el comportamiento vasodilatador observado debido a su limitada velocidad de difusión y su estabilidad en entornos ricos en hemoglobina.

Por otra parte, las NOS generan NO en presencia de O2 (o O2−·) y agua. Debido sus propiedades redox, el NO se puede oxidar para formar una serie de óxidos de nitrógeno (NOX), donde el N tiene estados de oxidación que varían del +1 al +5. Muchos de los estudios utilizados hasta la fecha utilizaban análisis o sensores que no eran selectivos para el NO, dando falsos positivos a la presencia y concentración de NO, cuando en realidad podría deberse a HNO, ONOO−, u otras especies reactivas de nitrógeno (RNS) derivadas del NO (Tennyson & Lippard 2011), lo que ampliaría la reactividad biológica del NO (Gautier-Sauvigné et al. 2005). Las RNS más importantes in vivo son N2O3 y ONOO− .(Wink & Mitchell 1998).

En la siguiente imagen se muestran los óxidos de nitrógeno en función del estado de oxidación del nitrógeno. En la tabla se resumen los nombres y las valencias de estas especies.

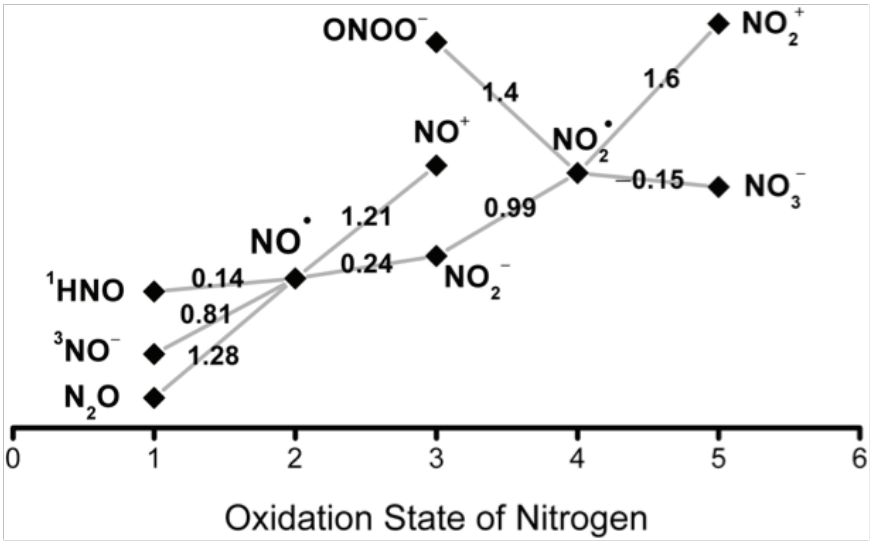


Imagen 6. Relaciones Redox entre especies NOx en función del estado de oxidación del nitrógeno (Tennyson & Lippard 2011). Los números sobre las líneas corresponden con los potenciales necesarios para cambiar el estado de oxidación.

Nombre	Símbolo	Valencia de N
Óxido nítrico	NO·	+2
Ión nitrosilo	NO–	+1
Ión nitrosonio	NO+	+3
Nitrito	NO_2–	+3
Peroxinitrito	ONOO–	+3
Peroxinitrito ácido	ONOOH	+3
Trióxido de dinitrógeno	N_2O_3	+2, +4
Dióxido de nitrógeno	NO_2·	+4
Tetraóxido de dinitrógeno	N_2O_4	+4
Nitrato	NO_3–	+5

Tabla 3. Óxido nítrico y especies relacionadas

Por lo tanto, reacciones que hasta ahora se consideraban mediadas por NO en realidad podrían deberse a estas otras especies relacionadas. Por todos estos motivos, en el estudio de la química del NO es fundamental realizar un estudio conjunto de todos las especies de nitrógeno implicadas, que se resume en la imagen 8 y la tabla 1.

N (+1): (NO-) Nitrosilo
HNO se forma por la reacción de un S-nitrosotiol con otro tiol, cuyos productos son HNO y disulfito (ver reacción 3).

EL HNO es tóxico a altas concentraciones, causando depleción del glutatión (GSH), pero también puede ejercer un papel beneficioso en el sistema cardiovascular, donde reacciona con dianas que no son reactivas al NO. Modula canales de calcio (Thomas et al. 2003).

N (+3): (NO_2–) Nitrito
Ver reacciones de formación 6, 7, 8, 14, 17, 21 y 29; esta última se produce en disolución acuosa a una velocidad próxima a la de difusión del NO.

Ver reactividad en 9 y 10 de la tabla. El NO_2– tiene funciones fisiológicas y bactericidas También se puede reducir por desoxihemoglobina o XOR para dar NO e ión hidróxido; la reacción con oxihemoglobina da como producto NO_3– (Tennyson & Lippard 2011).

N (+3): (ONOO–) Peroxinitrito
In vivo, el NO reacciona con superóxido (reacción 12), de manera limitada por la difusión del NO, para formar peroxinitritos. La química de los peroxinitritos es compleja. El anión peroxinitrito es una molécula bastante estable en solución alcalina. A pH<9, el anión peroxinitrito está en equilibro rápido con su conjugado, el peroxinitrito ácido (ONOOH) (pKa= 6.8) (reacción 20) (Trostchansky et al. 2010). La reactividad de los peroxinitritos se describe en las reacciones 13 a 19 de la tabla 1, y en la imagen 10. Ver reacción de NO con anión superóxido más adelante.

N (+4): ($NO_2^{\cdot}/N_2O_4$) Dióxido de nitrógeno

En presencia de O_2 el NO se auto oxida (reacción 4), ver reacción del NO con oxígeno más adelante. También se puede obtener por los procesos que se observan en las reacciones 9, 10, 15, 18, 25, 28 y 29.

El $NO_2^{\cdot}$ se auto oxida, dando un producto intermedio N_2O_4. En solución acuosa N_2O_4 hidroliza rápidamente para formar NO_2^- y NO_3^- (reacción 8). Ver también reacciones 5 y 19.

El $NO_2^{\cdot}$ es un oxidante fuerte, que se acumula durante las reacciones de oxidación de NO en solución acuosa y puede reaccionar con varias dianas a una velocidad relativamente alta. Al tener una molécula NO^+ puede actuar como agente nitrosante para generar organonitrosilos (Tennyson & Lippard 2011).

N (+2 y +4): (N_2O_3) Trióxido de dinitrógeno

El trióxido de dinitrógeno es una de las RNS más importantes in vivo. Se forma por la descomposición del ácido nitroso, y por la unión entre los radicales $NO^{\cdot}$ y $NO_2^{\cdot}$ (reacción 5). En condiciones fisiológicas las bajas concentraciones de NO hacen que éste difunda libremente, sin embargo, a concentraciones patológicas, el producto N_2O_3 alcanza niveles elevados (Bruckdorfer 2005).

Es una especie demasiado inestable en reacción acuosa para ejercer directamente ninguna función; Si no hay ninguna diana molecular, se hidroliza para formar NO_2^- (reacción 6). También se puede dar la reacción inversa, y sirve como fuente de NO y $NO_2^{\cdot}$ (reacción 5) (Tennyson & Lippard 2011).

Por otra parte, a pH fisiológico es el principal agente nitrosante, aunque no sea tan fuerte como el $NO_2^{\cdot}$, puede nitrosar los centros nucleofílicos de aminas (N-nitrosación), tioles (S-Nitrosación) y residuos hidroxilos (reacción 7) (Mariotto et al. 2004). Estas reacciones ocurren más rápidamente en medios lipídicos, como las membranas celulares, siendo factible en ciertas localizaciones. Los S-nitrosotioles son comunes in vivo, y hay evidencias que demuestran que funcionan como transportadores de NO, aunque los mecanismos no están claros (Bruckdorfer 2005; Reiter 2006).

Aminas, N-nitrosación: $N_2O_3 + RR'NH \rightarrow RR'NNO + H^+ + NO_2^-$

Residuos hidroxilos: $N_2O_3 + ROH \rightarrow RONO + H^+ + NO_2^-$

Tioles (S-nitrosación): $N_2O_3 + RSH \rightarrow RSNO + H^+ + NO_2^-$

N (+5): (NO_3^-) Nitrato

En humanos, la principal fuente de NO_3^- se produce por la ingesta de vegetales (60-80%); el resto proviene de las reacciones 1, 8, 13 y 24. Aunque para los humanos el NO_3^- sea inerte, las bacterias comensales lo utilizan como sustrato para conventirlo en NO_2^-, que pasa al estómago donde se puede transformar en diferentes RNS. Por lo tanto, todos los efectos relacionados con el NO_3^- se deben en realidad al NO_2^- u otras RNS (Tennyson & Lippard 2011).

5.2. Óxido nítrico (NO·), o monóxido de nitrógeno (N (+2))

Las reacciones en las que interactúa directamente el NO, y no otros óxidos de nitrógeno, importantes en su biología son aquellas que se producen a una velocidad fisiológicamente relevante. Serán éstas las reacciones con complejos metálicos de metales de transición y las reacciones con otros radicales libres. Otras reacciones en las que podría estar implicado el NO, como las reacciones con tioles y aminas, no son rápidas, y no tienen tanta relevancia en la bioquímica directa del NO (Wink & Mitchell 1998).

Reacciones directas del óxido nítrico

Reacciones con metales de transición		
	Nitrosilación	
	Oxidación	
	Reducción	
Reacciones con radicales		
	Reacciones con especies derivadas de oxígeno	
		Oxígeno
		Anión superóxido
		Radical hidroxilo
	Reacciones con lípidos	
	Reacciones con otros radicales	

Tabla 3. Reacciones directas del NO

5.2.1. Reacciones con metales de transición

El NO, por sus propiedades como radical, es capaz de donar electrones a los metales. El NO puede formar un enlace σ con el par de electrones del nitrógeno, y un enlace π con el electrón desapareado del $2p\pi^*$ con los electrones del orbital d de los metales de transición (Davis et al. 2001), actuando como donador de tres electrones. Así, reacciona con metales de transición para formar aductos metal-nitrosilo. Los metales de transición más importantes en sistemas biológicos son hierro, cobre y zinc. Estos metales son muy abundantes en los sitios catalíticos de los grupos prostéticos de enzimas y proteínas. Las reacciones con el hierro son las más estudiadas (Mariotto et al. 2004).

Como se muestra en la imagen, los principales tipos de reacciones por las que el NO reacciona con los metales de transición son:

a. Nitrosilación: reacción directa del NO con el centro metálico.
b. Oxidación: de complejos oxometálicos.
c. Reducción de oxo-complejos de valencias altas.

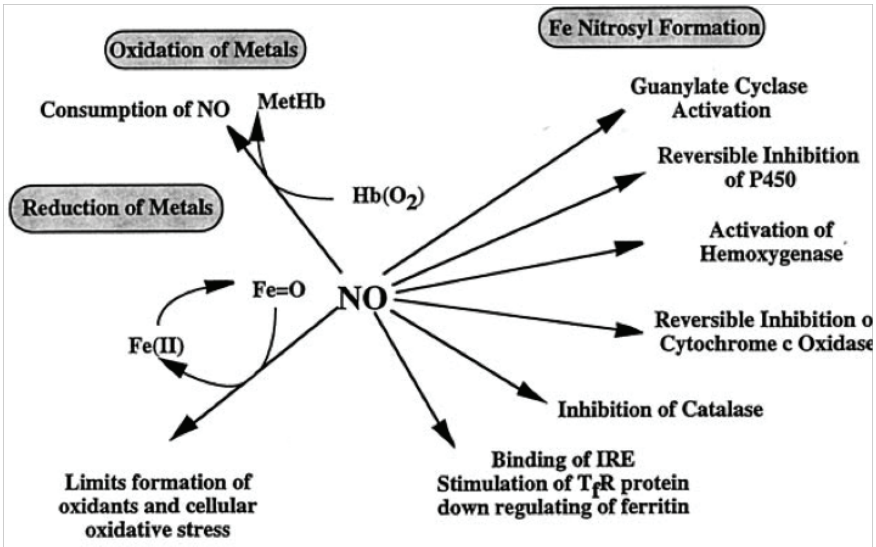


Imagen 7. Efectos directos del NO en metales de transición (Wink & Mitchell 1998)

Reacciones de nitrosilación

Se establecen interacciones covalentes con iones metálicos. Por ejemplo, el NO reacciona con ión ferroso (Fe2+) para formar complejos hierro-nitrosilo en proteínas que contienen el motivo hemo. Esta reacción no se produce cuando se trata de ión férrico (Fe3+). Las reacciones más importantes de este tipo se observan con la propia NOS, la sGC, el citocromo P450 (Wink & Mitchell 1998).

$$Fe^{aq}II + NO\cdot \rightarrow Fe^{aq}II{-}NO$$

Reacciones de oxidación

El NO también reacciona con complejos oxometálicos. Por ejemplo, reacciona rápidamente con metaloproteínas, como la oxihemoglobina. Dada la alta concentración de oxihemoglobina en la sangre, y lo rápido de la reacción con NO, este es la principal forma de control de la concentración de NO y uno de sus más importantes destinos metabólicos in vivo. En este contexto, la hemoglobina se considera no sólo uno de los principales "consumidores" de NO, sino también un almacén (Mariotto et al. 2004).

$$NO\cdot + Hb(Fe{-}O_2) \rightarrow MetHb(FeIII) + NO_3{-}$$

Reacciones de reducción

Este tipo de reacciones se produce con complejos metálicos que tienen valencias muy altas. Estas especies oxometálicas se han formado previamente por oxidación. La reacción con NO reduce la hipervalencia del complejo. El secuestro de especies oxometálicas peligrosas es una reacción importante mediante la cual el NO protege los tejidos del daño asociado a los peróxidos (Wink & Mitchell 1998).

$$NO\cdot + Fe{+}4{=}O \rightarrow Fe{+}3 + NO_2{-}$$

Reacciones con otros metales de transición

El NO también reacciona con moléculas que contengan otros metales de transición, como en los dominios de dedos de zinc.

5.2.2. Reacciones con radicales

El NO, al ser un radical con un electrón desapareado, reacciona con otros radicales libres como los formados con carbón, oxígeno y nitrógeno.

Reacciones con oxígeno molecular (O_2)

El NO es muy inestable en presencia de O_2. En solución acuosa, el NO reacciona con O_2 autooxidándose, dando lugar a NO_2· (reacción 4).(Ford et al. 1993).

En condiciones fisiológicas, el NO se produce a pequeñas concentraciones (del rango nM), y difunde rápidamente, como se ha visto anteriormente, reaccionando con sus dianas biológicas, por lo que los productos de la reacción de auto oxidación no son muy importantes. Sin embargo, en condiciones patológicas, cuando las concentraciones de NO aumentan significativamente (en el rango µM), esta reacción tiene gran importancia, ya que a su vez reacciona para dar N_2O_3, o NO_2^- y NO_3^-; (reacciones 5, 6 y 8) (Wink & Mitchell 1998).

La tasa de auto oxidación del NO depende sólo de la concentración de NO y O_2. Ambos son más solubles en entornos lipídicos que en soluciones acuosas (de 6 a 20 veces más) (Davis et al. 2001). Por tanto, las reacciones de nitrosación de tioles y aminas mediadas por N_2O_3 se darán principalmente en microentornos como las membranas, las cercanías de la NOS, o en núcleos hidrofóbicos de proteínas (Mariotto et al. 2004).

Reacciones con anión superóxido (O_2^{-}·)

La reacción entre el NO y el O_2^{-}· para producir el anión peroxinitrito ($ONOO^-$) es una de las más importantes de la química del NO. Es una reacción extremadamente rápida, cercana al límite de difusión (Trostchansky et al. 2010).

El principal mecanismo de pérdida de biodisponibilidad de NO es a través de la reacción con O_2^-. El O_2^- se genera por la reducción incompleta de un electrón del O_2 por la NADPH (fosfato de nicotinamida adenina dinucleótido reducido) o NADH (nicotinamida adenina dinucleótido reducido) oxidasas, principalmente por las enzimas de la cadena respiratoria de la mitocondrial (aproximadamente el 1% de la reducción del oxígeno). Estudios con animales de experimentación sugieren que muchas enfermedades vasculares están asociadas con un aumento de la formación de O_2^-.
Peroxinitritos y peroroxinitritos ácidos son potentes oxidantes, cediendo uno o dos electrones a varias moléculas, como tioles, súlfidos, proteínas, ADN y lípidos (Reiter 2006).Realizan sustituciones nucleofílicas para dar proteínas S-nitrosiladas y nitración de anillos aromáticos, en residuos de tirosina y alifáticos. La nitración de péptidos inducida por peroxinitritos reduce su fosforilación al exponerlos a ciertas quinasas, lo que sugiere que la nitración de las proteínas interfiere con las rutas de fosforilación. Además, inducen la activación de la guanilato ciclasa soluble (sGC), probablemente formando nitrosotioles. Los peroxinitri-

tos inician la peroxidación lipídica y reacciona directamente con ADN, impidiendo procesos de reparación o incrementando la producción de procesos genotóxicos, por lo que intervienen en muchos efectos patofisiológicos que van desde la inflamación hasta el cáncer. Además, el NO reacciona con radicales peroxilipídicos (LOO·) para acabar la peroxidación lipídica, produciendo peroxinitritos orgánicos.

Como las ROS son muy peligrosas, en la célula se dan muchos mecanismos enzimáticos que evitan su acumulación, como la superóxido dismutasa (SOD). En condiciones fisiológicas, el O_2^- reacciona con la SOD para formar H_2O_2 y agua, evitando que interaccione con el NO. Sin embargo, a altas concentraciones de O_2^-, el NO compite con la SOD por el O_2^-. La reacción está limitada por la difusión del NO, que es 6 veces más rápida que la dismutación del O_2^- por la SOD (Beckman & Koppenol 1996). Esto tiene dos efectos fisiológicos: por una parte disminuye la concentración de NO disponible, y por otra produce peroxinitritos.

Los peroxinitritos no son radicales libres, pero son a la vez ROS y RNS, lo que los convierte en potentes oxidantes.

Reacción con radical hidroxilo (OH·)
El NO también reacciona con el OH· a una velocidad próxima a la velocidad de difusión en solución acuosa (reacción 7).

Reacción con radicales alquilo (R·), alcoxi (RO·) y alquilperoxi (ROO·)
El NO reacciona rápidamente con radicales alquilo (R·), alcoxi (RO·) y alquilperoxi (ROO·).

Este tipo de reacciones son críticas en la regulación de la peroxidación lipídica catalizada por metales o enzimas y la capacidad del NO para sensibilizar células hipóxicas de mamífero frente a la radiación.

$$R\cdot + NO\cdot \rightarrow RNO$$
$$RO\cdot + NO\cdot \rightarrow RONO$$
$$ROO\cdot + NO\cdot \rightarrow ROONO$$

La peroxidación lipídica es un proceso patológico de daño a tejidos que se da en diferentes enfermedades. Es una reacción en cadena, donde se forma radicales lipídicos alcoxi (LO·) o alquilperoxi (LOO·). EL NO reacciona rápidamente con estos radicales. Como el producto obtenido no es un radical libre, la reacción en cadena se detendría en estos productos, limitando la peroxidación lipídica en distintas enfermedades, como la aterosclerosis (Mariotto et al. 2004).

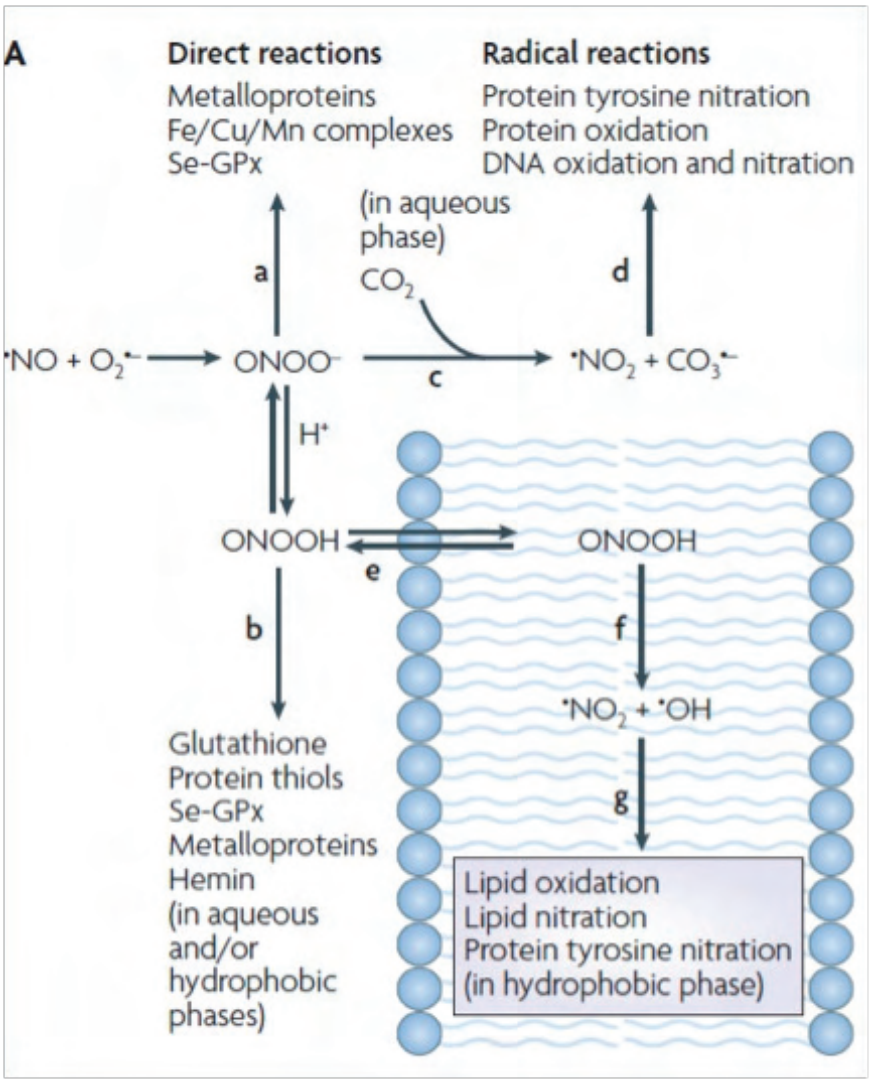


Imagen 8 Bioquímica de los peroxinitritos: dianas y destinos (Szabó et al. 2007).

6. División funcional del estudio del NO: efectos directos e indirectos en función de la concentración.

Una división funcional de los mecanismos de acción del NO divide sus reacciones en directas o indirectas. Las reacciones directas son aquellas en las que el NO reacciona directamente con sus dianas moleculares, a bajas concentraciones. Reacciones indirectas son aquellas que cursan a través de RNS o de otras moléculas que son a su vez dianas biológicas, a altas concentraciones. La concentración de NO depende de los "determinantes cinéticos de interacciones con dianas moleculares": velocidad de producción, distancia de difusión, velocidad de consumo y reactividad de los RNS con sus dianas moleculares. Las variadas funciones del NO se explican por las dianas moleculares que alcanza a cada concentración.

Las reacciones directas son aquellas en las que el NO reacciona directamente con sus dianas moleculares, a concentraciones fisiológicas (rango nM). Generalmente en estas condiciones el NO es poco reactivo. Este tipo de reacciones incluyen las reacciones con metales, la S-nitrosilación, y también las reacciones con radicales libres, explicadas anteriormente. Las bajas concentraciones de NO, por debajo de los 200nM, favorecen las reacciones directas con proteínas con un grupo hemo, metaloenzimas y otros radicales libres (Reiter 2006).

Reacciones indirectas son aquellas que cursan a través de RNS o de otras moléculas que son a su vez dianas biológicas, como se ha explicado anteriormente. Serán estas especies, y no el NO, las que alcancen las dianas biológicas finales. Las reacciones indirectas se pueden subdividir en química nitrosativa y oxidativa, e incluyen los siguientes mecanismos: nitrosación, nitración y oxidación (Wink & Mitchell 1998). La nitrosación in vivo está mediada principalmente por N_2O_3, mientras que la oxidación lo hace por otras RNS, como $ONOO^-$, $NO_2^{\cdot}$ y HNO (Thomas et al. 2003). Las reacciones indirectas se dan a concentraciones de NO relativamente altas, por encima de los 400 nM.

Muchas veces se asocian las reacciones directas a bajas concentraciones de NO con reacciones fisiológicas, y las reacciones indirectas a altas concentraciones de NO con reacciones patológicas o deletéreas. Esta clasificación no siempre corresponde con la realidad, pues las reacciones mediadas por RNS también pueden ser importantes reacciones señalizadoras. Por ejemplo, la nitrosación de las caspasas produce su inhibición enzimática y, por consiguiente, previene la apoptosis.

Las células y tejidos que producen NO pueden seguir tanto reacciones directas como indirectas; sin embargo, las células o tejidos lejanos al lugar de producción sólo pueden seguir reacciones di-

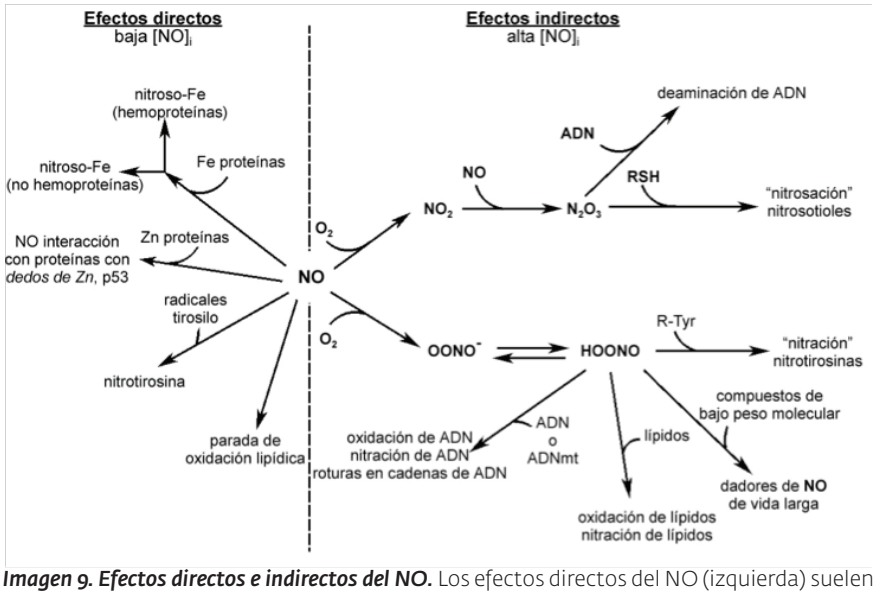


Imagen 9. Efectos directos e indirectos del NO. Los efectos directos del NO (izquierda) suelen estar producidos por bajas concentraciones de NO, mientras que los efectos indirectos (derecha) son producidos por altas concentraciones de NO. (Davis et al. 2001)

rectas, ya que el NO alcanza estos sitios por difusión, reaccionando por el camino con muchas otras moléculas, y llegando a los tejidos distantes sólo a muy bajas concentraciones. Además, las dianas biológicas del NO varían también en función del tiempo de exposición a esta molécula. Se ha observado que algunas proteínas responden de manera inmediata al NO, mientras que otras necesitan de horas o días (Thomas et al. 2009).

Para estudiar en detalle la relación de estas respuestas biológicas con la concentración y el tiempo de exposición al NO, se utilizan conocidos donadores de NO, aproximación que se realizará también en esta tesis.

6.1. Factores que afectan a la concentración: determinantes cinéticos de interacciones con dianas moleculares

La concentración de NO depende, a su vez, de un conjunto de factores denominados "Determinantes cinéticos de interacciones con dianas moleculares". Estos factores comprenden la velocidad de producción de NO, la distancia de difusión, la velocidad de consumo y la reactividad de los RNS con sus dianas moleculares (Thomas et al. 2010; Laranjinha et al. 2012).

Formación de NO

Este tema se trata en profundidad en las secciones dedicadas a la formación de NO y la regulación de las NOS.

Difusión de NO

Para hacernos una idea de lo que significa la difusión del NO, supongamos que las células tienen un tamaño aproximado de entre 50-300 um. Debido a su velocidad de difusión, el NO difundirá en un segundo la distancia similar a cinco-diez células. La concentración de NO disminuye en función de la distancia, desplazándose en un volumen de una esfera. Así, cuando el NO difunde una distancia (r), el volumen aumentará proporcionalmente al cubo de la distancia. Dado que la concentración se expresa en moles/volumen, cuando el NO difunde a una distancia de una célula (r=2), el volumen de la esfera aumentará ocho, y su concentración se diluirá por ocho; si la distancia fuera de cuatro células (r=5), la concentración se dividiría por 125, y así sucesivamente. Así, después de tan sólo un segundo de su producción, la concentración del NO se puede haber diluido hasta 200 veces simplemente por difusión.

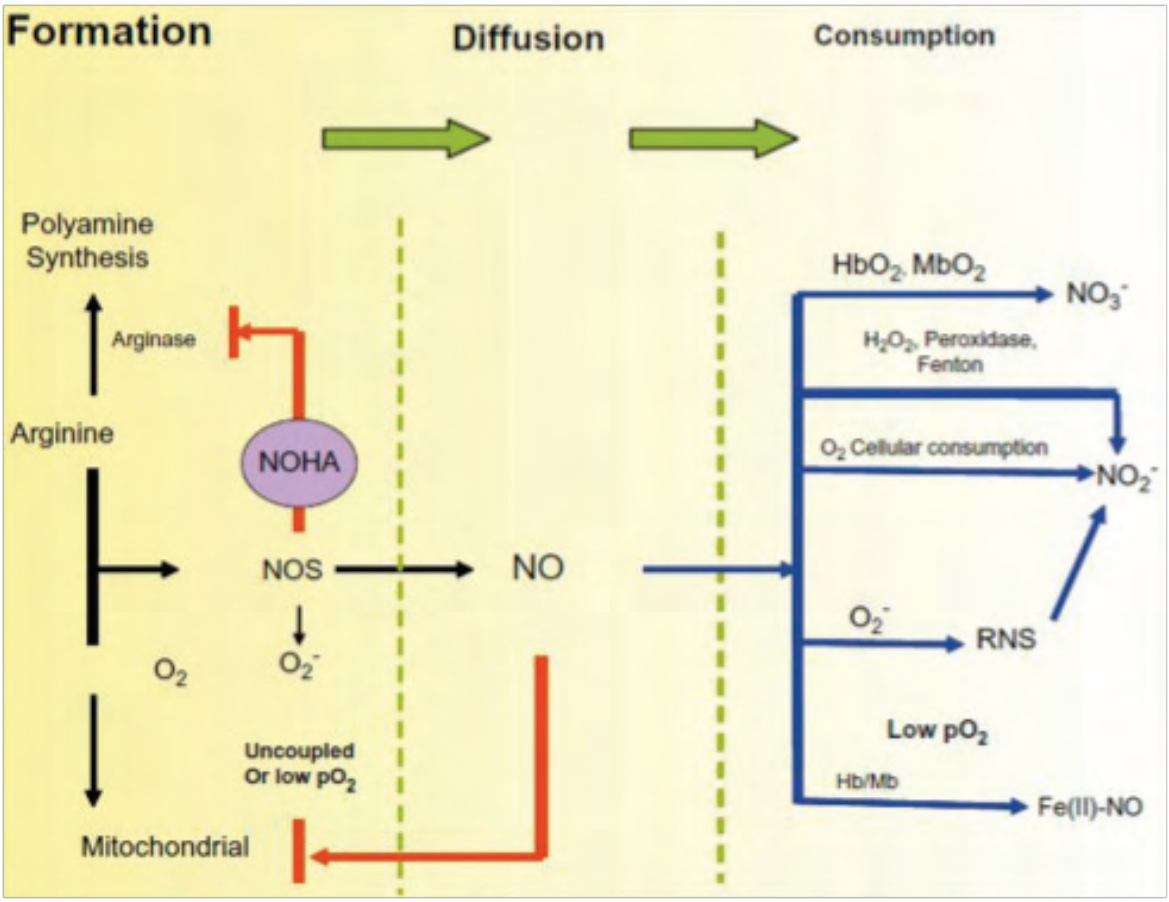


Imagen 10. Determinantes cinéticos de interacción con dianas moleculares del NO. Formación, difusión y consumo. Los determinantes cinéticos influyen en la señalización dependiente de la concentración de NO in vivo (Thomas et al. 2010)

Difusión de NO

La difusión es un factor físico determinante de la concentración de NO.

El NO es una molécula no cargada muy pequeña, soluble en medios acuosos e hidrofóbicos. Tiene una capacidad de difusión mayor que el oxígeno. Precisamente esta facilidad de difusión hace que sea difícil mantener una concentración de NO alta, no muy lejos del lugar donde se está produciendo, incluso dentro de una misma célula.

Esto implica que la exposición al NO es diferente en función de la localización, incluso dentro de la misma célula. Por ejemplo, para la estimulación de la liberación de catecolaminas en una región sináptica separada menos de 1um, se requiere una concentración de NO 1uM; Sin embargo, esta concentración de NO será de sólo 1nm a una célula de distancia. La fosforilación de p53 necesita de una concentración estable de NO superior a 400nM. En tejido humano inflamado la iNOS (óxido nítrico sintasa inducible) colocaliza con fosfo-p53, para alcanzar una concentración de NO en ese microentorno (Thomas et al. 2010).

Consumo
Los principales mecanismos de consumo de NO son dependientes de O2. Las interacciones fisiológicas más importantes se dan con las células de la serie roja, ROS, y con sus dianas moleculares.

6.2. Dianas moleculares en función de la concentración

El NO está implicado en infinidad de procesos fisiológicos y patológicos, a veces incluso realizando funciones opuestas (por ejemplo, el NO puede ser un factor pro-apoptótico y anti-apoptótico).

Una explicación sencilla a esta dualidad es que la activación de diferentes rutas de señalización se produce a diferentes concentraciones de NO. En la imagen desarrollada por Wink (Wink et al. 2011) se pueden observar diferentes niveles de actividad. Los procesos mediados por cGMP se producen a bajas concentraciones de NO ([NO] < 1–30 nM). Los demás reacciones tienen lugar a concentraciones crecientes de NO: fosforilación de Akt (proteína quinasa B) ([NO] = 30–100 nM), estabilización de HIF-1a ([NO] = 100–300 nM), fosforilación de p53 ([NO] > 400 nM), y estrés nitrosativo ([NO] > 1 uM). Cabe destacar también, como se ha explicado anteriormente, que la concentración de NO a la que están sometidas las células varía mucho en función de la distancia a la que se encuentre de la fuente de NO, y el tiempo de exposición.

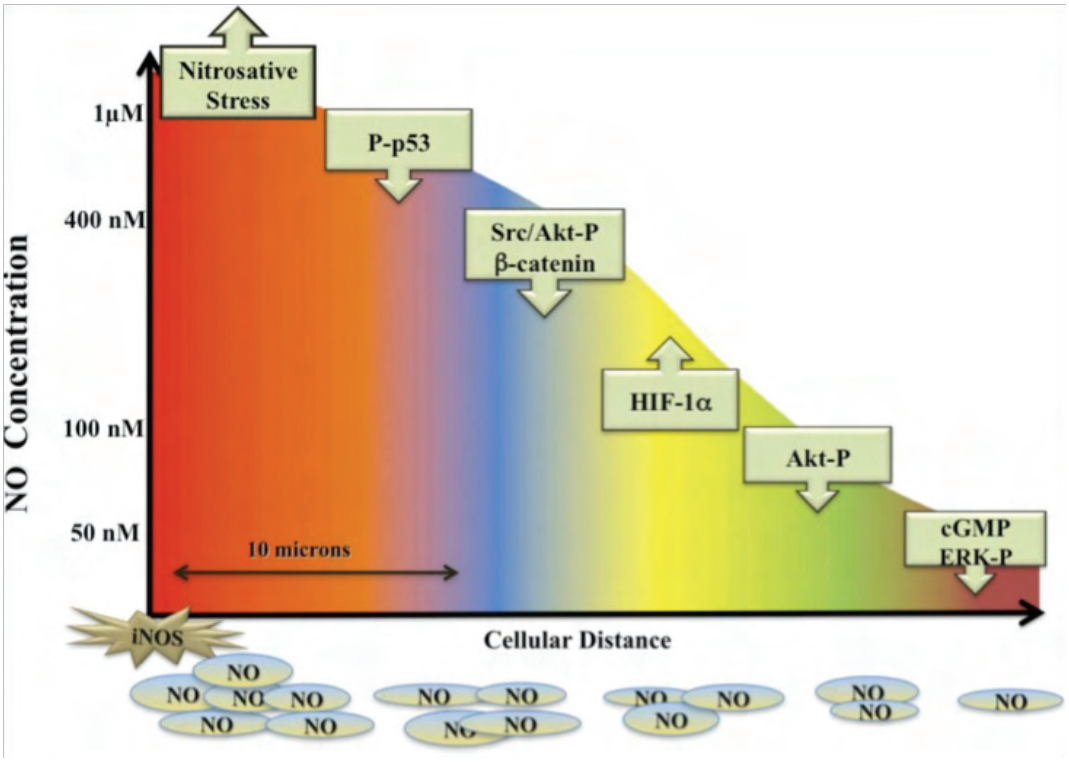


Imagen 11. Concentraciones sostenidas de NO y mecanismos de transducción de señales implicados (Wink et al. 2011). La imagen representa los niveles de NO necesarios para activar diferentes rutas de señalización en células tumorales o endoteliales. Los datos se obtuvieron al tratar las células durante 24 horas con DETA-NO (Diethylenetriamine NONOate). El eje de abscisas representa la distancia entre las células y una fuente de NO (un macrófago murino activado, con iNOS produciendo NO > 1 uM, in vitro).

Guanilato ciclasa soluble

La reacción del NO con el grupo hemo de la sGC es la más importante de la química del NO. La reacción entre el NO y la sGC produce un complejo Fe-nitrosilo que se activa para formar cGMP, un segundo mensajero fundamental. Por la importancia de la sGC en los procesos de regulación de la neurotransmisión, neurosecreción, plasticidad sináptica, vasodilatación y agregación plaquetaria y relajación del músculo liso, se considera que la mayor parte de los efectos biológicos del NO están mediados por la interacción con esta proteína.

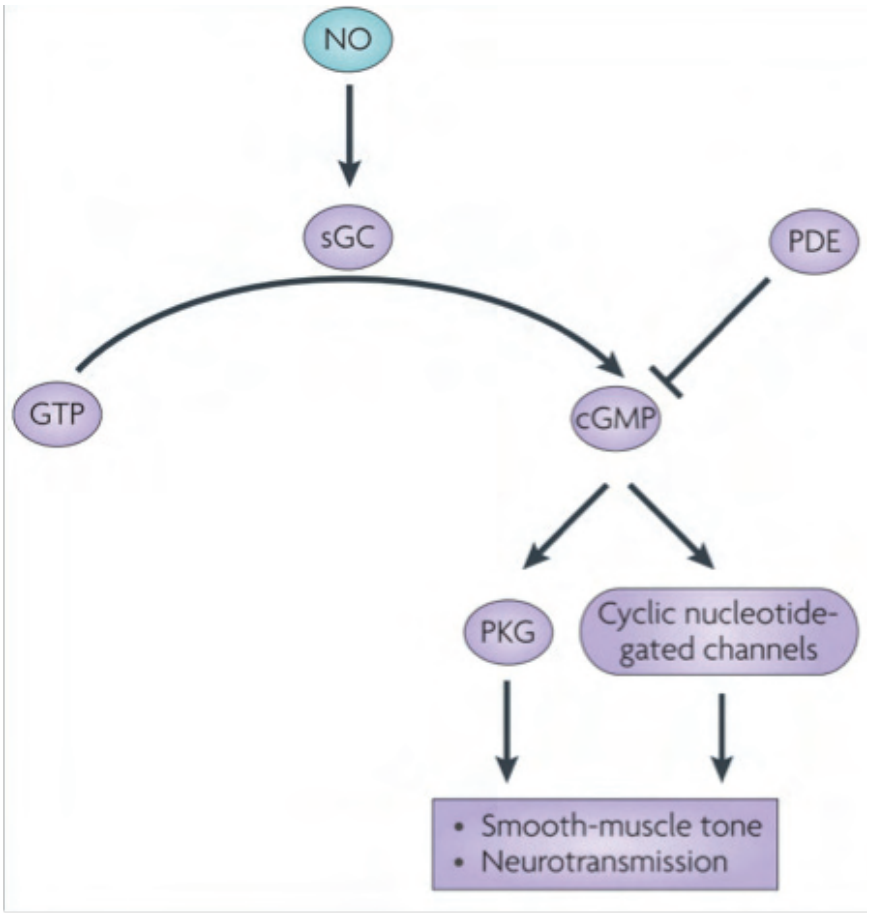


Imagen 12. El NO activa la sGC. La sGC es una enzima citosólica con un grupo hemo, que cataliza la transformación de guanosina trifosfato (GTP) en cGMP. La sGC se activa por la unión del NO al grupo hemo, aumentando la concentración intracelular de cGMP. El cGMP actúa a su vez sobre varias moléculas, como la PKG y los canales de compuerta de nucleótidos cíclicos. Por estas vías el NO ejerce sus funciones de neurotransmisor y como relajante en la musculatura lisa. La fosfodiesterasa (PDE) hidroliza el cGMP, impidiendo una acumulación excesiva (Calabrese et al. 2007).

6.3. Concentraciones de NO *in vivo*, y comparación con resultados *in vitro*.

Los niveles de NO que se dan in vivo no se han terminado de esclarecer, pero en general se sabe que la nNOS y la eNOS producen niveles bajos, en el rango de 0,2 a 2,0 nM (Hall & Garthwaite 2009), mientras que el NO producido por la iNOS puede ir de los 2-20 nM, hasta concentraciones de 200 nM (Björne et al. 2005).

Muchos de los resultados obtenidos se deben al uso de donadores de NO. Estas prácticas (también utilizadas en esta tesis) entrañan ciertos peligros. Por un lado, las concentraciones de NO liberado por sus donadores no siempre corresponden con las encontradas in vivo. Por ejemplo, las equivalencias entre DETA-NO y NO son: 40–60 µM DETA-NO = 50 nM NO; 80–120 µM DETA-NO = 100 nM NO; 500 µM DETA-NO = 400 nM NO; y 1 mM DETA-NO = 1 µM NO (Wink et al. 2011). Por otro lado, los donadores son a veces capaces de producir efectos fisiológicos por sí mismos, como es el caso de los peroxinitritos o del DETA-NO (Thompson et al. 2009).

Brown destacaba, en un artículo reciente (Brown 2010), que las concentraciones de NO utilizadas para provocar la muerte celular in vitro podrían encontrarse con los siguientes problemas al compararse con datos in vivo: a) Los altos niveles de NO utilizados in vitro podrían no tener una correlación con los menores niveles de NO encontrados in vivo; b) se inhibiría la respiración mitocondrial, con múltiples efectos, y c) en presencia de oxígeno, superóxido o tioles, tales concentraciones de NO generarían rápidamente RNS. Brown sugería comparar estos resultados con los producidos con las NOS in vitro, aunque destacaba que estos datos también podrían no ser correctos, ya que las concentraciones de NO podrían ser superiores a las que se dan in vivo, al faltar los principales consumidores de NO (hemoglobina y mioglobina).

7. Funciones del NO

La nNOS está principalmente implicada en la señalización neuronal, la neurotoxicidad, la plasticidad sináptica y la modulación de rutas implicadas en el aprendizaje y la expresión del dolor.

Las funciones del NO son numerosas, y dependen en gran medida de la isoforma que lo produzca. En la imagen 16 se resumen las funciones fisiológicas y patológicas en las que está implicado.

Las principales funciones de la eNOS son la regulación de la función vascular. El NO promueve la vasodilatación e inhibe la agregación plaquetaria y leucocitaria, así como la interacción entre plaquetas y leucocitos en el endotelio vascular. También atenúa la respiración mitocondrial, la apoptosis, y el estrés oxidativo. Los efectos del NO sobre la mitocondria, la muerte celular y el daño oxidativo protegen el corazón y otros tejidos frente a los daños isquémicos. El NO también aumenta la respuesta angiogénica tras una isquemia prolongada. En condiciones isquémicas, el NO_2^- se reduce a NO confiriendo citoprotección, mejora del flujo sanguíneo y angiogénesis vascular (Kevil & Lefer 2010).

Las grandes cantidades de NO producidas por la iNOS pueden tener efectos beneficiosos en su función microbiocida, antiviral, antiparasitaria y antitumoral. Por otro lado, una inducción aberrante de la iNOS puede ser patofisiológica, produciendo asma, artritis, esclerosis múltiple (EM), colitis, psoriasis, enfermedades neurodegenerativas, desarrollo de tumores, rechazo a los trasplantes o choque séptico (Pautz et al. 2010).

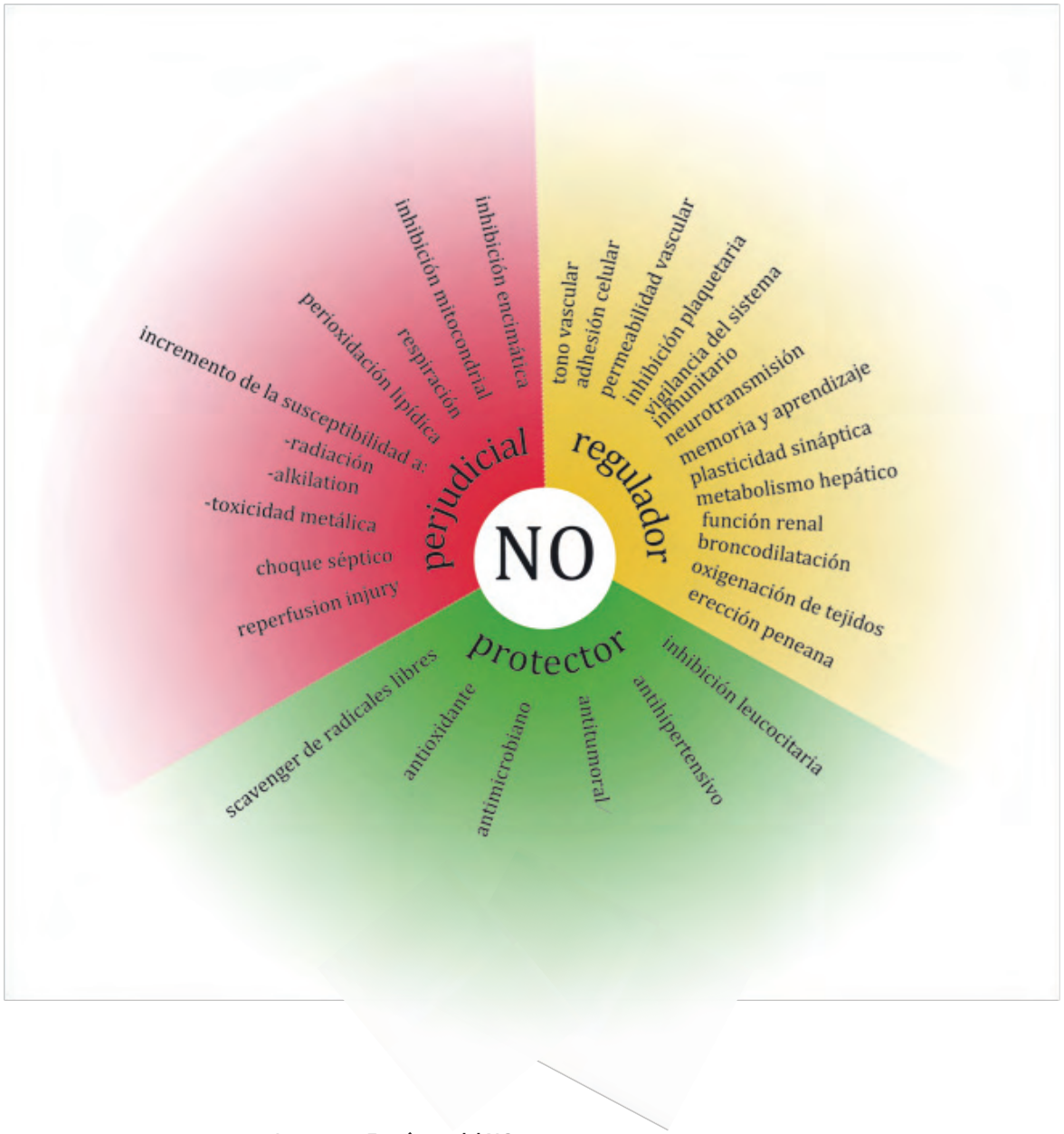


Imagen 13. Funciones del NO

7.1. NO en el Sistema Nervioso Central

Las tres isoformas de la NOS se encuentran tanto en el SNC, pero las principales funciones fisiológicas relacionadas con el NO se deben a la acción del nNOS (Esplugues 2002).

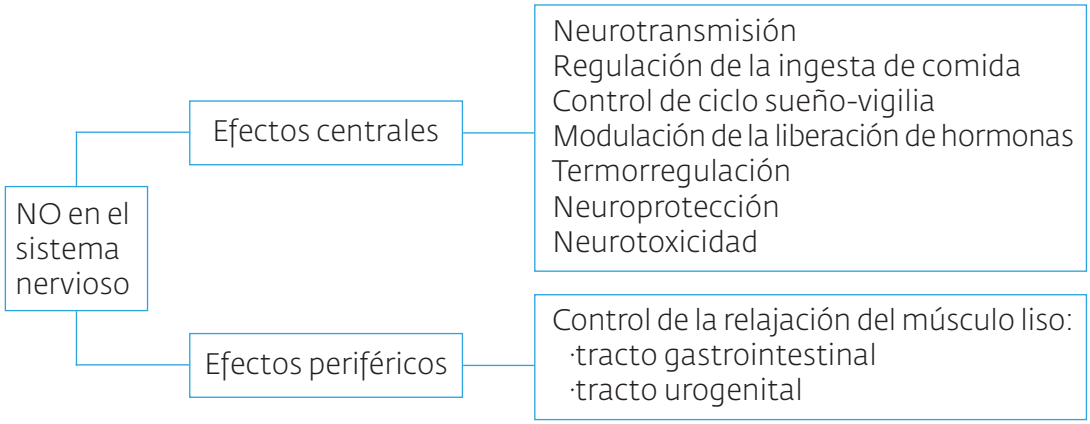


Imagen 14. El NO en el sistema nervioso central y periférico.
Adaptación de (Calabrese et al. 2007)

La señalización por NO

El NO interacciona con sGC estimulando su actividad. El aumento de los niveles intracelulares de cGMP influye en la plasticidad sináptica, la relajación del músculo liso, la neurosecreción y la neurotransmisión. El NO también interacciona con hemoproteínas como la ciclooxigenasa y la hemo oxigenasa 1. Esta familia de proteínas está implicada en respuestas metabólicas, inflamatorias y de estrés celular. El NO regula la vía de la quinasa Akt y el factor de transcripción CREB (cyclic-AMP-responsive-element- binding protein), dos vías que promueven la supervivencia celular y la neuroprotección ante el estrés. Además, el NO regula la señalización celular mediante la S-nitrosilación, mediante la cual se une covalentemente a los grupos tioles de proteínas y de otras moléculas. Esta reacción puede ser tanto neuroprotectora como neurotóxica (Calabrese et al. 2007).

Implicación en funciones centrales y periféricas

El NO está relacionado con el desarrollo del cerebro, la formación de la memoria y el comportamiento mediante la regulación de la plasticidad sináptica. La inhibición de la síntesis de NO produce amnesia y afecta el aprendizaje espacial y la memoria olfativa y disminuye la actividad locomotora en tareas de habituación. El NO también está implicado en el desarrollo del cerebro, el procesamiento visual, el aprendizaje discriminativo, el comportamiento frente a la comida y la bebida, la termorregulación, la tolerancia y abstinencia a opiáceos, ritmos circadianos, sueño, generación de patrones respiratorios...

Neurogénesis

La neurogénesis es un proceso importante en el desarrollo del sistema nervioso que, contrario a lo que se pensaba clásicamente, se sigue produciendo en el cerebro adulto de manera natural, por ejemplo tras un accidente cerebrovascular, o ataques epilépticos.

La neurogénesis en el cerebro adulto se observa principalmente en la zona subventricular y en la zona subgranular del giro dentado, con presencia de nNOS y precursores neuronales de la nNOS, respectivamente. En general se sugiere que el NO endógeno derivado de la nNOS tiene un papel antiproliferativo en la neurogénesis de la zona subventricular de los animales adultos, aunque su acción en el hipocampo es controvertid. Por contra, la administración de donadores de NO promueve la neurogénesis (Zhou & Zhu 2009).

Modulación de la plasticidad sináptica

El NO es un mensajero retrógrado que coordina el aumento de los mecanismos pre y post-sinápticos involucrados en las principales formas de plasticidad sináptica: potenciación a largo plazo (PLP, LTP en inglés) y depresión a largo plazo (DLP, LTD en inglés).

En la inducción de la PLP participa el glutamato. El glutamato actúa sobre sus receptores AMPA (Ácido a-amino-3-hidroxi-5-metil-4-isoxazolpropiónico) y NMDA (N-Metil-D-Aspartato), activando una serie de eventos en los que participan la Ca2+-CaMK (proteína quinasa dependiente de calcio-calmodulina), las NOS y varias proteínas quinasas. La PLP se correlaciona con la memoria y el aprendizaje, y se observa principalmente en las centros cerebrales superiores, implicados en las funciones cognitivas, en particular en la corteza cerebral y el hipocampo. En estudios in vitro, la inhibición de las NOS evitaba el desarrollo de la PLP. Las isoformas implicadas en la PLP son la nNOS y la eNOS presente en las neuronas, la sGC y CaMK.

La DLP está mediada por la activación de PKC (proteína quinasa dependiente de calcio) y PKG y de las rutas de NO independientes de cGMP. La DLP se observa en las regiones superiores del cerebro, aunque se ha estudiado mucho en el cerebelo, donde se ha propuesto como un modelo de aprendizaje de movimientos motores (Esplugues 2002).

Percepción del dolor

El NO está implicado en las vías neurales nociceptivas centrales y periféricas (Mao 1999). Funcionalmente, la mayoría de los reflejos nociceptivos implican la interacción de NO con receptores de NMDA (NMDAR). El papel de NO cambia en función del estímulo doloroso. La inhibición del NO tiene un efecto antinociceptivo cuando el dolor proviene de terminales nerviosas estimuladas químicamente, en modelos te hiperalgesia térmica y dolor visceral. Por otro lado, el bloqueo de la síntesis de NO exacerba el dolor en modelos de hiperalgesia mecánica. La implicación del NO en los efectos antinociceptivos de las drogas también es controvertida (Esplugues 2002).

Depresión

La depresión será la primera causa de incapacidad en el 2020, según estimaciones de la OMS. El NO producido por la NOS juega un papel fundamental en la depresión. Se ha observado que las concentraciones plasmáticas de nitritos aumentan significativamente en pacientes con depresión. Además, los antidepresivos imipramina y paroxetina inhiben la actividad de la NOS tanto en animales como en humanos (Zhou & Zhu 2009).

Enfermedades del SNC

El NO está relacionado con la etiología de enfermedades neurológicas, como enfermedades neurodegenerativas crónicas y autoinmunes.

Las concentraciones de NO presentes en el tejido inflamado bloquean reversiblemente la conducción en axones normales, desmielinizados y remilienizados. En enfermedades que se caracterizan por una pérdida generalizada de mielina, acompañadas de inflamación, como la EM y el síndrome de Guillain-Barre, las altas concentraciones de NO podrían exacerbar los síntomas neuronales tanto en el SNC como en el SNP.

El NO está implicado en la muerte neuronal tras un trauma. En la médula espinal, tras la avulsión de las raíces de los nervios espinales se observa la expresión de la nNOS y, posteriormente la muerte de las motoneuronas. Un pre-tratamiento con inhibidores de la nNOS aumenta sustancialmente la supervivencia neuronal.

La excesiva liberación de glutamato y NO, junto con el estrés oxidativo y la disfunción mitocondrial están relacionadas con varias enfermedades neurodegenerativas. Tras un ataque epiléptico, se induce la nNOS en varias regiones corticales (Esplugues 2002).

El Parkinson se asocia con una progresiva pérdida de neuronas dopaminérgicas en la sustancia negra, que conlleva disfunción motora extrapiramidal, con temblores, rigidez y bradiquinesia. Se ha observado que los pacientes con Parkinson presentan una mayor producción de NO y de nitración de tirosinas, así como una sobreexpresión de la nNOS en los ganglios basales. Además, se ha observado en modelos de Parkinson que los ratones knock-out para nNOS, y los tratados con inhibidores de las NOS son más resistentes a la neurotoxicidad y es protector en modelos de enfermedad de Huntington.

En la enfermedad de Alzheimer se ha observado un gran aumento de la expresión de las tres isoformas de las NOS (Zhou & Zhu 2009).

La administración de metales (aluminio y mercurio) induce cambios en la actividad de la nNOS en el cerebro y el cerebelo, lo que sugiere que la nNOS es un mediador en las enfermedades cerebrales inducidas por metales.

7.2. NO en el Sistema Nervioso Periférico

En el SNP, el NO es un importante neurotransmisor inhibitorio de los neuronas no colinérgicas no adrenérgicas (NANC). Los nervios nitrérgicos periféricos se distribuyen ampliamente, y producen la relajación del músculo liso en los sistemas gastrointestinal, respiratorio, vascular y urogenital.

Esta relajación inducida por el NO produce en el sistema genital, en los cuerpos cavernosos, la erección peneana. En el sistema gastrointestinal, el NO permite la acomodación de grandes volúmenes de comida en el estómago sin aumentar significativamente la presión intraluminal, regula el tono muscular de los esfínteres intestinales y permite el peristaltismo del tracto gastrointestinal. En el sistema pulmonar, el NO funciona como broncodilatador. En el sistema vascular la nNOS se encuentra en los nervios perivasculares de varios vasos sanguíneos, y constituye un mecanismo de control regional del flujo de sangre alternativo e independiente de la eNOS. Este NO parece ser particularmente relevante en la regulación del flujo sanguíneo cerebral. En el músculo esquelético hay una gran expresión de la nNOS, principalmente bajo el sarcolema de fibras rápidas, enfatizando el papel del NO como modulador de la fuerza contráctil (Esplugues 2002; Calabrese et al. 2007).

8. El NO y su papel en apoptosis

8.1. Apoptosis

La apoptosis es un tipo de muerte celular programada, una muerte activa. Es una ruta que apenas ha sufrido cambios con la evolución, que en condiciones patológicas produce un proceso inflamatorio y requiere la inversión de energía por parte de la célula para llevarse a cabo. Entre sus características, se dan un conjunto de alteraciones morfológicas: reducción del tamaño, pérdida de contacto con las células vecinas, formación de vacuolas citoplasmáticas, condensación de la cromatina en la membrana nuclear y formación de cuerpos apoptóticos (fragmentos de núcleos condensados rodeados por una pequeña parte del citoplasma). La membrana celular se mantiene íntegra en todo momento, pero sin embargo ocurren muchos cambios en su superficie, el más típico el "flipping" de fosfatidilserinas de la cara interna a la externa de la membrana. Hay también una serie de determinantes bioquímicos que facilitan el reconocimiento de este tipo de suicidio celular: se produce un 25-70% de depleción de ATP (adenosina trifosfato), el contenido de calcio intracelular incrementa en el rango de 200-400 nM, con una progresiva depleción de GSH, inhibición de Bcl-2 (B-cell lymphoma/leukemia-2), cursando todo ello con la ayuda de caspasas (Jellinger 2001).

El NO juega un papel dual en la generación/prevención de la apoptosis. Por un lado, se ha demostrado su implicación como agente pro-apoptótico en la activación de receptores de muerte, generación de peroxinitritos, inhibición de la síntesis de ATP mitocondrial, e inactivación de enzimas antioxidantes; Por otra parte, también se ha demostrado su papel anti-apoptótico

La apoptosis se puede iniciar de muchas maneras que en general se clasifican en dos grupos según su mecanismo de acción: 1-Los que se unen directamente a la maquinaria de muerte, 2-Los que lo hacen indirectamente. En la primera categoría se encuentran polipéptidos homólogos al factor de necrosis tumoral -α (TNF-α) y el receptor de muerte CD95 (CD95/Fas), que se unen físicamente a las caspasas en unos dominios denominados dominios de muerte, iniciando así la cascada apoptótica. En la segunda categoría se encuentran los estímulos que activan indirectamente las caspasas, bien regulando la bioquímica de los dominios de muerte, bien a través de otras rutas celulares. Por otro lado, la apoptosis puede cursar vía mitocondria, a través de la activación de la cascada de caspasas. La clasificación más utilizada en los procesos apoptóticos se basa en su dependencia/independencia de cGMP (Leist & Nicotera 1998; McConkey 1998).

8.2. El NO y su papel en la apoptosis

El NO juega un papel dual en la generación/prevención de la apoptosis. Por un lado, se ha demostrado su implicación como agente pro-apoptótico en la activación de receptores de muerte, generación de peroxinitritos, inhibición de la síntesis de ATP mitocondrial, e inactivación de enzimas antioxidantes; Por otra parte, también se ha demostrado su papel anti-apoptótico. Los mecanismos por los que el NO protege de muerte celular se dividen clásicamente en dependientes/independientes de cGMP (Nicotera et al. 1997; Nicotera et al. 1999; Brüne et al. 1998; Brüne et al. 1999; Brüne 2003; Brüne 2005; Curtin et al. 2002; Krumenacker et al. 2004)

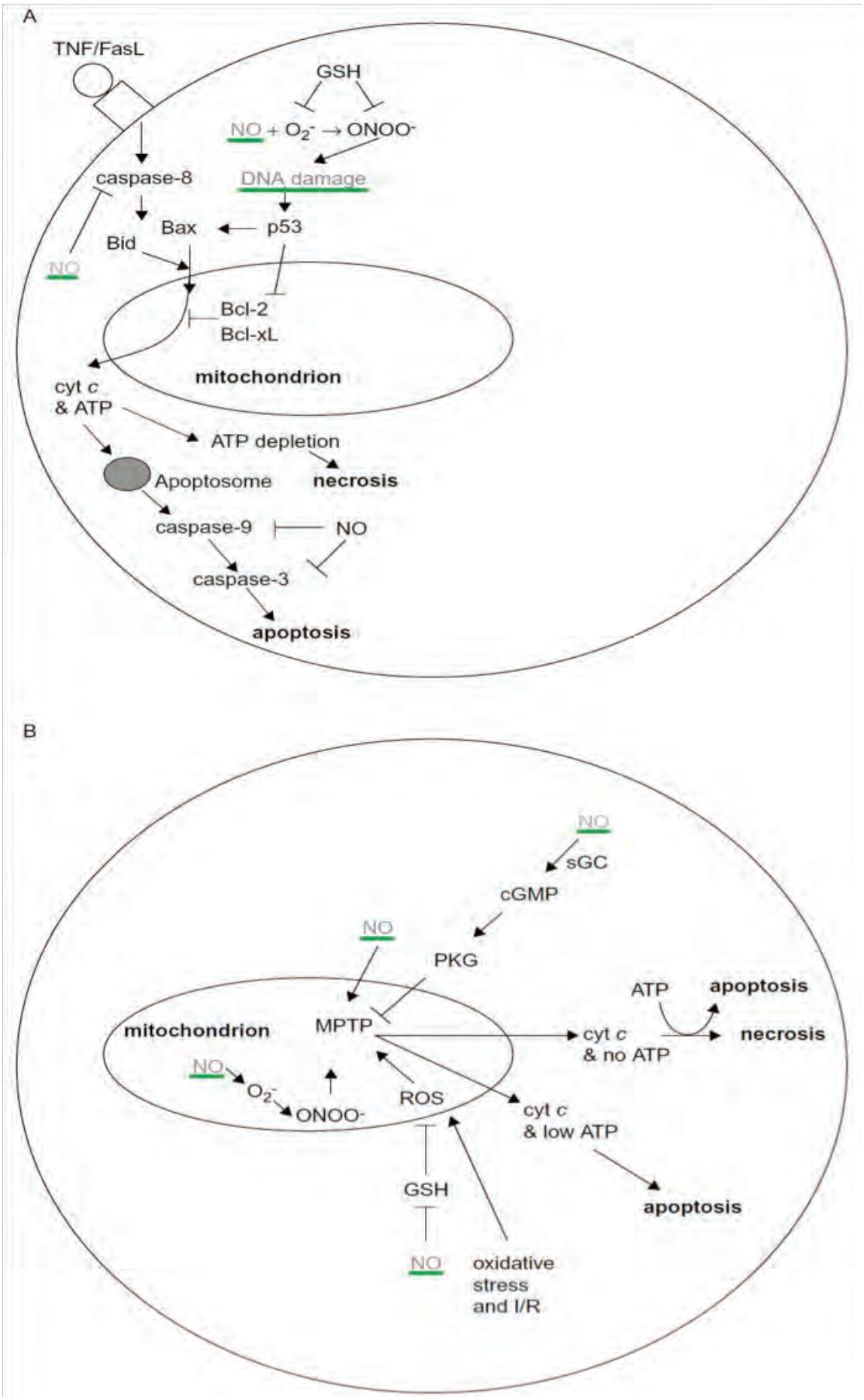

A
TNF/FasL
GSH
NO + O_2^- → $ONOO^-$
caspase-8
DNA damage
Bax
p53
Bid
NO
Bcl-2
Bcl-xL
mitochondrion
cyt c
& ATP
ATP depletion
Apoptosome
necrosis
caspase-9
NO
caspase-3
apoptosis
B
NO
sGC
cGMP
NO
PKG
ATP
apoptosis
mitochondrion
MPTP
cyt c
& no ATP
necrosis
NO
O_2^-
ONOO-
ROS
cyt c
& low ATP
GSH
apoptosis
NO
oxidative
stress
and I/R

Imagen 15. Efectos pro-apoptóticos y anti-apoptóticos (verde) del NO (Loughran et al. 2010). **A)** Efectos del NO mediados por Bax (proteína X asociada a Bcl-2). Los poros de Bax pueden provocar la salida de cit c de la mitocondria hacia el citosol. En presencia de ATP, se forma el apoptosoma y se produce apoptosis. Si no hay ATP se observa necrosis. Los efectos antiapoptóticos del NO en esta ruta están mediados por la S-nitrosación de las caspasas 3, 8 y 9, inhibiéndolas. Los efectos pro-apoptóticos del NO en esta vía están mediados por peroxinitritos, con el consiguiente daño al ADN y regulación positiva de p53. **B)** Efectos del NO mediados por el MPTP. Los MPTP pueden mediar la salida del cit C de la mitocondria al citosol, con las reacciones previamente descritas. El papel anti-apoptótico del NO se produce por la generación de cGMP y subsecuente activación de PKG, suprimiendo el MPTP. El papel pro-apoptótico del NO está mediado por la regulación positiva del MPTP, induciendo directamente el poro por vía NO o peroxinitrito, o inhibiendo la GSH, que actúa inhibiendo los ROS producidos por el estrés oxidativo y la isquemia/reperfusión (I/R).

8.2.1. Papel del NO pro-apoptótico

Como se ha visto anteriormente, altas concentraciones de NO y/o peroxinitritos inducen apoptosis en muchos tipos celulares (macrófagos, timocitos, islotes pancreáticos, tumores y neuronas). La activación de las rutas de muerte por NO es muy variada, pero en general es independiente de cGMP.

Ruta mitocondrial

El NO se une a la cit c oxidasa del complejo IV de la mitocondria, generando superóxido que, en estas condiciones, reacciona con NO para producir peroxinitritos, que inducen una pérdida en el potencial de membrana, permitiendo la liberación de cit c al citosol y activando la ruta de las caspasas.

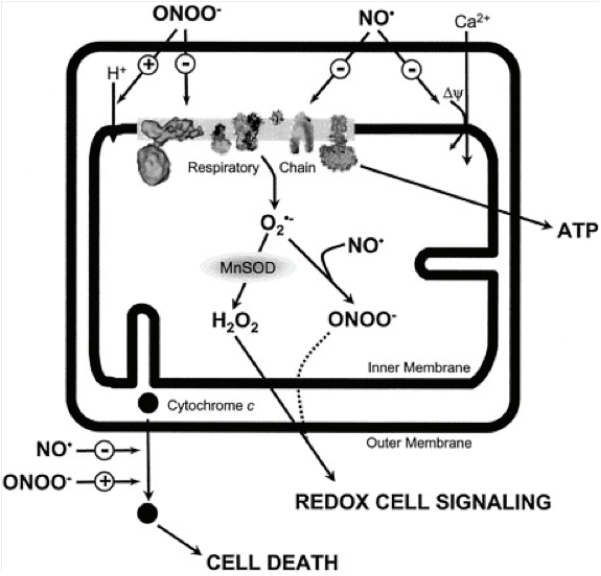


Imagen 16. Modelo de ruta de señalización NO mitocondrial-citocromo c oxidasa (Brookes et al. 2002). El NO inhibe primariamente la citocromo c oxidasa (complejo IV de la cadena de transporte electrónico mitocondrial), lo que favorece la generación de O2- por la cadena respiratoria, sin que la síntesis de ATP se vea afectada. El O2- es dismutado por la Mn-SOD, produciendo H2O2 en la matriz mitocondrial. El H2O2 es capaz de atravesar las membranas y participar, así, en procesos de señalización rédox en el citosol. Alternativamente, el O2•- puede reaccionar con el NO para producir ONOO-. Este poderoso oxidante inhibe distintos complejos de la cadena respiratoria y aumenta la permeabilidad de la membrana mitocondrial interna a protones. El ONOO- también desencadena la liberación de citocromo c, activando la muerte por apoptosis. Algunos autores sostienen que el NO es capaz de inhibir la liberación de citocromo c, actuando, probablemente, sobre la captación mitocondrial de Ca2+.

Ruta de las caspasas por inducción de p53
El NO y los peroxinitritos son capaces de dañar el ADN celular, promoviendo una acumulación de p53, que detiene a su vez el ciclo celular actuando de manera dependiente a la ruta de las caspasas, al promover la liberación de cit c y aumentar el ratio Bax/Bcl-xL (Bcl-xL: variante de ayuste alternativo larga de Bcl-x).

Activación de JNK/SAPK
NO activa el grupo de MAPK (proteínas quinasas activadas por mitógenos) denominado JNK/SAPK (Proteína quinasa N-terminal de Jun), mediadores de la activación de caspasa 3 inducida por NO. La activación dependiente de NO de la caspasa 3, de JNK/SAPK y de p38 MAPK induce muerte celular por apoptosis. La supresión de las actividades JNK/SAPK y p38 MAPK mediante transfección de PKC en macrófagos RAW 264.7 protege a estas células de la apoptosis inducida por el donador de NO nitroprusiato de sodio. Esta acción inhibidora ocurre vía inhibición de la actividad de caspasa 3 (Jun et al. 1999), e indica que JNK/SAPK y p38 MAPK pueden tener una función importante en la activación de caspasa 3 inducida por NO.

Apoptosis mediada por la generación de ceramida
El NO aumenta la concentración de ceramida, que induce la activación de caspasas 3 y 9, liberación de cit c, activación de JNK/SAPK, y supresión de Bcl-2.

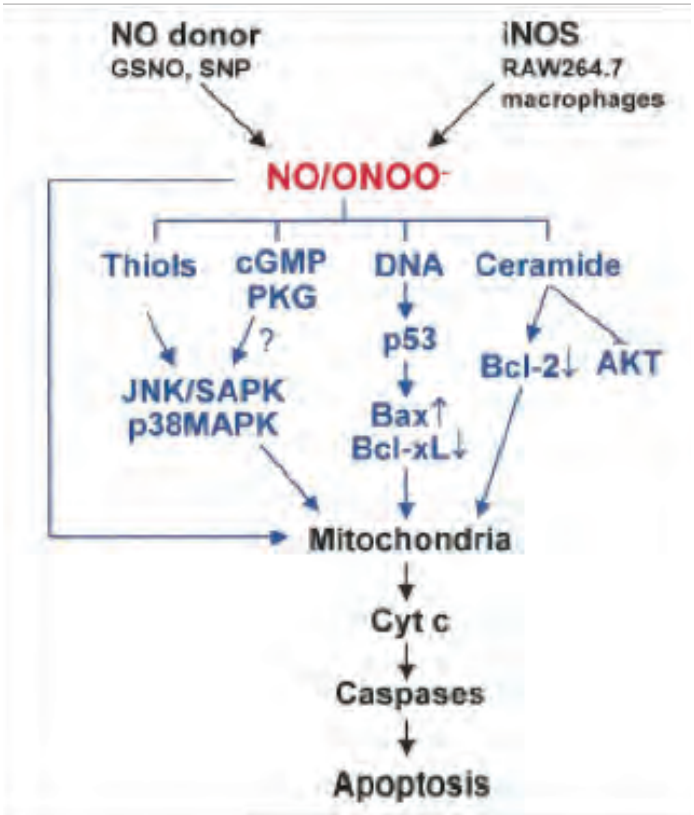


Imagen 17 Cascada de señales pro-apoptóticas inducida por el NO (Chung et al. 2001)

8.2.2. Papel del NO anti-apoptótico

El NO juega también un papel anti-apoptótico en las neuronas y células cromafines, protegiendo a la célula de la apoptosis inducida por muchos factores, como TNFα, estrés oxidativo, y la privación de suero o glucosa.

Inhibición vía NO/cGMP

El mecanismo por el que NO protege de la apoptosis a través de cGMP no está claro. Se cree que estimula la producción de cGMP y que éste inhibe muchas de las rutas apoptóticas mencionadas arriba: inhibe la liberación al citosol de cit c, la producción de ceramida y la activación de las caspasas, aumenta los niveles de Bcl-2, induce la fosforilación de Bad (Bcl-2 antagonist of cell death) y procaspasa 9, y activa NF-κB (factor nuclear kB).

S-nitrosilación

La S-nitrosilación inactiva la actividad de la caspasa, impidiendo el correcto desarrollo de la activación de esta ruta. Esta inactivación se produce cuando el NO S-nitrosila la cisteína que contienen las caspasas en su sitio catalítico. Por otra parte, la S-nitrosilación es también un modulador del estado redox de la célula, inhibiendo la excesiva activación del NMDAR.

Regulación de genes anti-apoptóticos

NO actúa también en la inducción de la expresión de muchos genes anti-apoptóticos.

NO^+ (nitrosación) Recciones nitrosantes, X-NO	**$RS(H) + X\text{-}NO \rightarrow RS\text{-}NO + X^- + H^+$**	**Dianas**	**Función propuesta**
Acidificación	$NO_2^- + H^+ \rightarrow HO\text{-}NO$	Vías aéreas Tejido Isquémico Intestino	Broncodilatación Protector en apoptosis Actividad antimicrobiana
$NO\cdot/O_2$	$NO\cdot + O_2 \rightarrow ONO\text{-}NO\ (N_2O_3)$ $\rightarrow O_2NO\text{-}NO\ (N_2O_4)$	RyR? / proteínas de membrana Albúmina Sérica	Contracción de músculo estriado Homeostasis de la presión sanguínea
$NO\cdot/O_2^-$ (si $[NO] \gg [O_2^-]$)	$NO\cdot + O_2^- \rightarrow ONO\text{-}NO\ (N_2O_3)$	Sustrato de NOS	Señalización de NOS Detoxificación $NO\cdot/O_2^-$
Catalizadas por metales	$NO\cdot + M^{n+} \rightarrow M^{n+}\text{-}NO$ $NO_2^- + 2M^{n+} \rightarrow M^{n+}\text{-}NO + M^{n-1}$	Cys93 de Hb (eritrocitos) Glutation plásmico Cys93 de Hb (eritrocitos)	Vasodilatación hipóxica, inhibición plaquetaria Estímulo ventilatorio Vasodilatación hipóxica
Transnitrosilación	$RS\text{-}NO$ $(RS)_2Fe\text{-}(NO)_2$	AE1 (R=Hb) Desconocida	Vasodilatación hipóxica Niveles SNO en estado estacionario
Reacciones NO·	**$RS^-/RS\cdot + NO\cdot \rightarrow RS\text{-}NO$**	**Dianas**	**Función propuesta**
Radical-radical	$RS\cdot + NO\cdot \rightarrow RS\text{-}NO$	Glutation Albúmina Sérica RyR?	Estrés oxidativo Vasodilatación, agregación plaquetaria ¿Contracción muscular?
Radical-intermedio	$RS^- + NO\cdot \rightarrow [RS\text{-}NO]^- + Y$ $\rightarrow RS\text{-}NO + Y^-$	RyR?	¿Contracción muscular?

Tabla 4. Mecanismos de la S-nitrosilación y sus implicaciones fisiológicas (adaptado de (Foster et al. 2003)

9. El NO y el glutamato

Existe una relación física entre el NO y el glutamato. La nNOS se encuentra en el interior de las membranas postsinápticas, anclada al receptor ionotrópico de glutamato de tipo NMDA Esta unión tiene un significado fisiológico muy importante. La actividad de la nNOS aumenta por la elevación de la concentración de calcio intracelular (Bredt 1996), por un mecanismo dependiente de Ca2+-CaM Tras la unión de glutamato al NMDAR, éste se abre y permite la entrada de calcio, aumentando la actividad de la nNOS. Este mecanismo explica por qué muchos de los efectos del glutamato estén mediados por el NO.

Los receptores de glutamato se clasifican en ionotrópicos y metabotrópicos. Los ionotrópicos se subclasifican a la vez en receptores tipo NMDA, KA y AMPA. El glutamato se une a estos receptores glutamatérgicos e inicia una vía de señalización post-sináptica. Existe una relación física entre el NO y el glutamato. La nNOS se encuentra en el interior de las membranas postsinápticas, anclada al receptor ionotrópico de glutamato de tipo NMDA. La unión entre receptor y nNOS no es directa. La nNOS está unida por el extremo amino terminal (N-terminal) con el dominio PDZ (PSD-95 Discs large/ZO-1homology) de la proteína PSD-95 (Post-Synaptic Density protein 95), que a su vez está unida al NMDAR. De este modo, PSD-95 concentra la presencia de las nNOS cerca del NMDAR (Christopherson et al. 1999).

Esta unión tiene un significado fisiológico muy importante. La actividad de la nNOS aumenta por la elevación de la concentración de calcio intracelular (Bredt 1996), por un mecanismo dependiente de Ca^{2+}-CaM (Guix et al. 2005). Tras la unión de glutamato al NMDAR, éste se abre y permite la entrada de calcio, aumentando la actividad de la nNOS. Este mecanismo explica, además, por qué muchos de los efectos del glutamato estén mediados por el NO.

La excitotoxicidad es una liberación excesiva de glutamato en la sinapsis, o una sobre estimulación de sus receptores, que provoca daño o muerte neuronal. El NMDAR es el receptor glutamatérgico más relacionado con la excitotoxicidad por glutamato y el daño neuronal tras una isquemia focal, debido a su gran permeabilidad al Ca2+. La sobre estimulación del NMDAR produce una entrada masiva de Ca2+ en las neuronas postsinápticas, activando enzimas dependientes de Ca2+, como la nNOS (Zhou & Zhu 2009), y la superproducción de NO.

El NO aumenta el daño cerebral al reducir la producción de energía inhibiendo las enzimas mitocondriales y glicolíticas, aumentando el daño al ADN, y generando peroxinitritos que incrementan los niveles de radicales libres (Small et al. 1999).

Varios estudios *in vitro* implican al NO procedente de la nNOS como la principal fuente de neurotoxicidad neuronal. En cultivos primarios de neuronas corticales, la eliminación transgénica de la nNOS hacía que estas células fueran resistentes a la neurotoxicidad mediada por NMDA. Los cultivos neuronales knock-out para nNOS son más resistentes a la falta de oxígeno y glucosa. En modelos animales de isquemia focal, la activación de la nNOS está relacionada con el daño neuronal de accidente cerebrovascular (Zhou & Zhu 2009).

Por el contrario, el NO producido por la activación de la eNOS juega un papel protector en la isquemia cerebral al mantener el flujo

de sangre a la región cerebral. La inhibición de la nNOS con concentraciones de inhibidores de NOS que no suprimían la actividad de la eNOS reducían el volumen infartado, mientras que el uso de inhibidores selectivos de la nNOS es neuroprotector en modelos animales de isquemia focal. El daño se aumenta al inhibir la eNOS a altas concentraciones de inhibidores no selectivos de las NOS, con alteraciones perjudiciales del flujo de sangre cerebral y subsecuente aumento del volumen infartado (Esplugues 2002).

Las interacciones y los mecanismos de señalización relacionados con estas vías son complejos. La protección vascular está relacionada con mecanismos mediados por cGMP. La inhibición de la sGC y de la producción de cGMP previene la neurotoxicidad mediada por glutamato y NO (Montoliu et al. 1999). La s-nitrosilación de GSH por el NO se ha implicado en el sistema de defensa neuronal antioxidativo, ya que el NO parece atrapar las especies reactivas de oxigeno y compensa parcialmente el daño oxidativo inducido por la isquemia (Wink et al. 2011). Así mismo, el NO podría tener una función neuroprotectora interaccionando directamente con el NMDAR, disminuyendo la unión del glutamato, y por tanto disminuyendo el flujo de Ca2+a través del canal tras su activación.

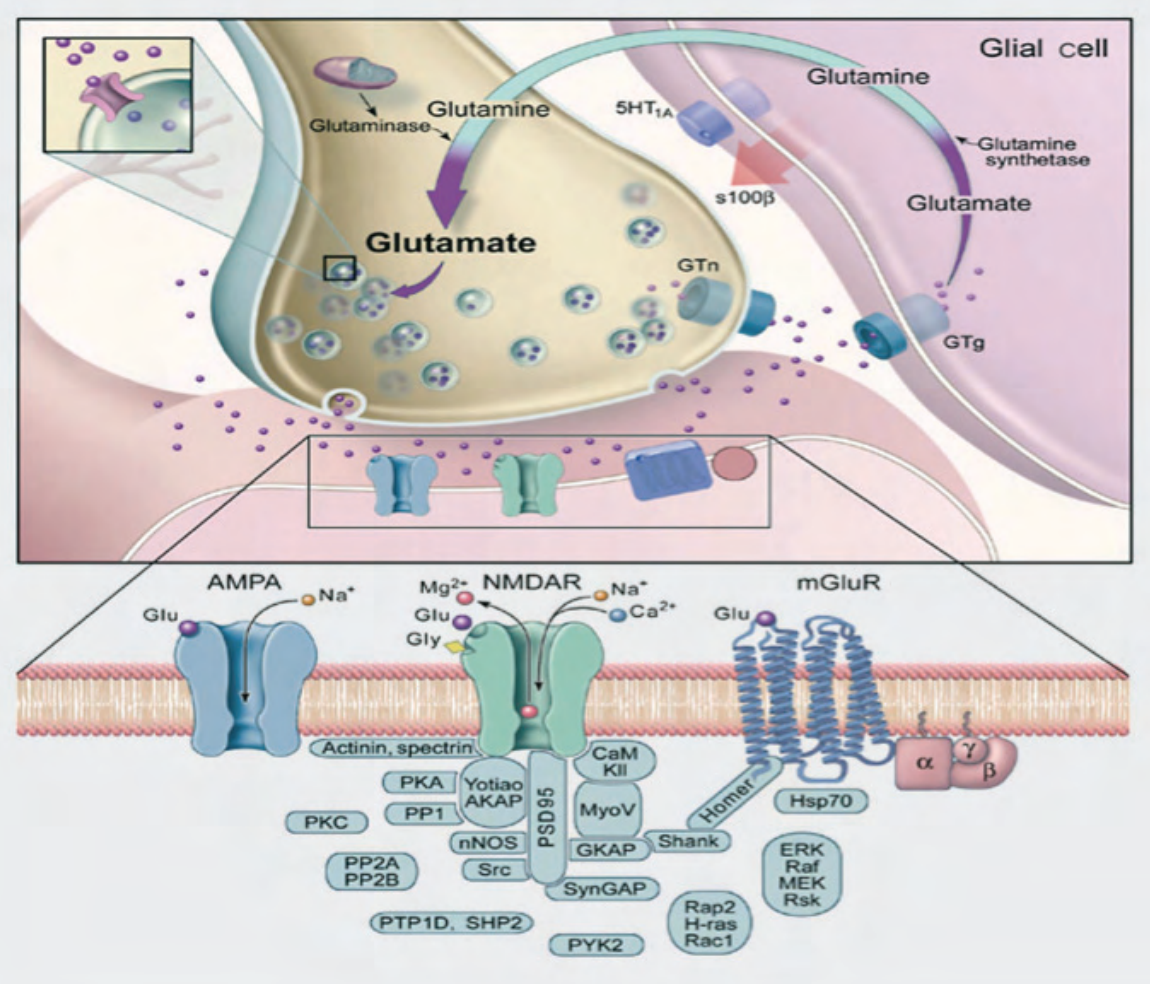


Imagen 18. Relación entre el sistema glutamatérgico y el NO (Szabo et al. 2003)

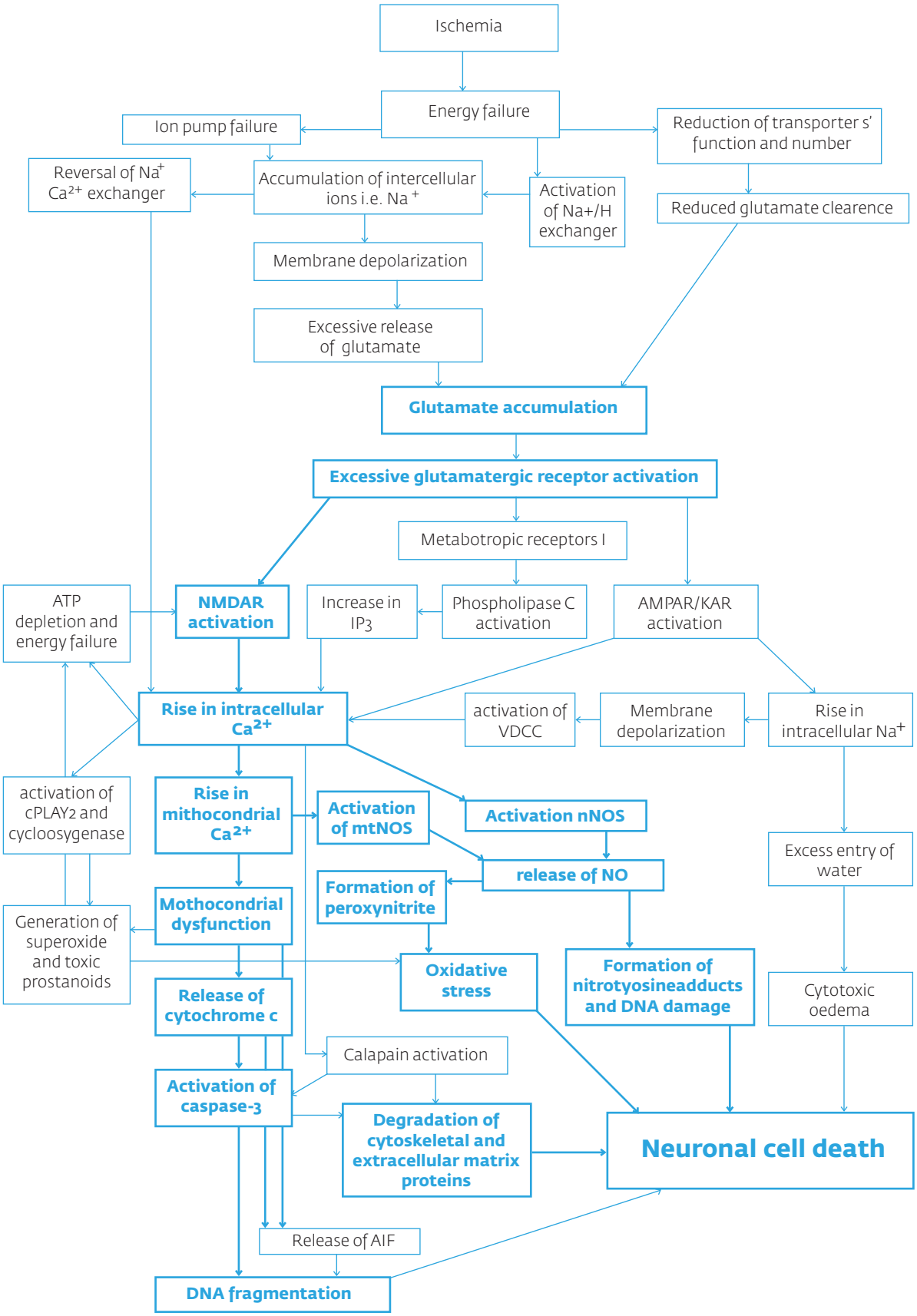


Imagen 19. Resumen del papel del glutamato y el NO durante la isquemia neuronal (Kostandy 2012)

La generación de peroxinitrito es el principal mediador citotóxico en el daño inducido por glutamato. El aumento de la producción de peroxinitritos aumenta la concentración de calcio citosólico, contribuyendo a la muerte neuronal. El NO y los ROS generados pueden causar peroxidación lipídica y daño a las membranas. La producción de peroxinitrito se ha observado en cerebros postisquémicos. El daño causado por NO y $ONOO^-$ al ADN es un importante mecanismo neurotóxico. Además, la inhibición de las enzimas de la cadena respiratoria mitocondrial exacerba la disminución de los almacenes de energía neuronal. Las altas concentraciones locales de NO pueden reducir la viabilidad celular nitrosilación varias enzimas, como la PKC y la gliceraldehido-3-fosfato deshidrogenasa, o interaccionando con el hierro presente en el grupo hemo o los complejos no hemo asociados con enzimas como la P450.

Las condiciones inflamatorias y los periodos de isquemia transitoria inducen la expresión de la iNOS en varias poblaciones de células cerebrales. Sin embargo, la expresión de la iNOS se da después de la de la nNOS o la eNOS, y el sitio celular de esta expresión depende de la naturaleza de la lesión. Los neutrófilos presentan inmunoreactividad para la iNOS en el cerebro tras isquemia permanente, y es predominante en las células vasculares en isquemia permanente, y es abundante en astrocitos reactivos de la isquemia global. El NO producido por la iNOS es perjudicial en el cerebro isquémico, contribuye a la progresión del daño tisular y aumenta la neurotoxicidad ejercida por glutamato (Esplugues 2002).

B. Óxido nítrico sintasas

La síntesis endógena de NO se ha demostrado en prácticamente todos los tipos celulares y todos los órganos (Geller & Billiar 1998). Esta síntesis está catalizada por una familia de enzimas denominadas NOS. En los mamíferos se han descubierto hasta el momento tres isoformas diferentes de las NOS: NOS neuronal, nNOS o NOS 1; NOS endotelial, eNOS o NOS 3; NOS inducible, iNOS o NOS 2; más una cuarta isoforma en la mitocondria, la NOS mitocondrial o mtNOS, sobre la que todavía no hay un claro consenso. El nombre de las isoformas proviene de las células en las que se descubrió por primera vez, y el número corresponde al orden en el que fueron aisladas. Todas ellas difieren en su función, secuencia de aminoácidos, regulación (Ferrero & Torres 2002; Haynes et al. 2004) y genes de procedencia (Krumenacker et al. 2004).

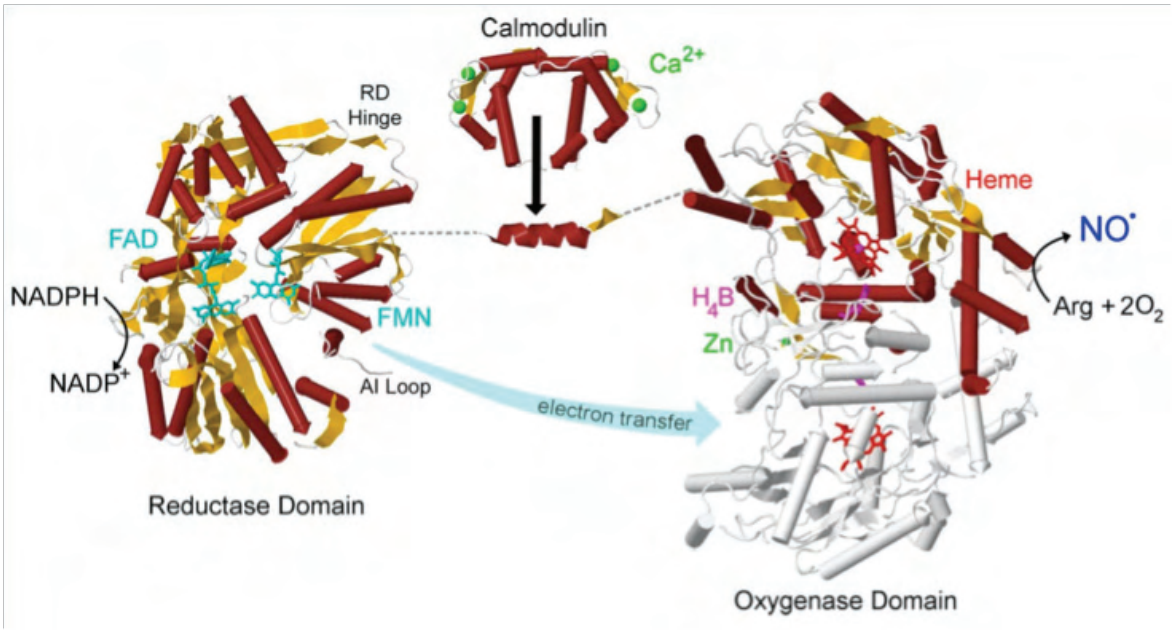


Imagen 22. Estructura de los dominios de las NOS alineados según su secuencia de aminoácidos. Domino reductasa a la derecha, dominio de unión a CaM en el centro abajo, y dominio oxigenasa a la izquierda, y la CaM (centro, arriba) (Daff 2010).

1. Isoformas de la NOS

Las tres principales isoformas de la NOS (nNOS, eNOS e iNOS) ejercen diferentes funciones en el organismo. Dos de estas isoformas, la nNOS y la eNOS, se expresan de manera constitutiva en los tejidos, son dependientes de Ca2+ y producen NO en pequeñas cantidades (en el rango nM) que ejercen funciones fisiológicas y citoprotectoras. La tercera isoforma, la iNOS, se expresa principalmente tras ser inducida (aunque se ha observado su expresión constitutiva en algún tipo celular) por estímulos tales como las citoquinas, no depende de Ca2+ y produce cantidades de NO en el rango uM. Cuando las NOS constitutivas se activan por largos periodos de tiempo, o cuando se activa la iNOS, se producen altas concentraciones de NO y peroxinitritos, potencialmente peligrosas. Para realizar lecturas más extensas se recomienda (J. Liu et al. 1995; Y.-W. Kwon et al. 2003; Domenico 2004).

Gen humano	Tipo celular	Cromosoma	Tamaño Gen	Estructura Gen
nNOS	Neuronal Músculo esquelético Músculo cardiaco	12; 12q24.2-12q24.3	160 kb	29 exones, 28 intrones
eNOS	Endotelial	7; 17cen-q11.2	21 kb	26 exones, 25 intrones
iNOS	Casi todos	17; 7q35-7q36	37 kb	26 exones, 25 intrones

Tabla 5. Estructura de los genes y localización cromosómica de las NOS humanas (Geller & Billiar 1998; Alderton et al. 2001)

nNOS

Se encuentra principalmente en las neuronas del SNC, aunque también se observa, entre otros, en nervios periféricos y músculo esquelético. Su estructura presenta zonas de unión para mononucleótido de flavina (FMN), dinucleótido de flavina adenina (FAD), NADPH, CaM, proteínas quinasas A y C (PKA y PKC), y un dominio característico, exclusivo de esta isoforma: el dominio PDZ. Necesita la unión de Ca2+-CaM para ser activa. Se regula por transcripción, transducción y fosforilación. Esta isoforma constitutiva está implicada en procesos de neurotransmisión, plasticidad neuronal, memoria, e hiperalgesia. Interviene en la función eréctil y la regulación del tránsito intestinal. Su hiperactividad produce citotoxicidad y disfunción y muerte neuronal.

eNOS

Está presente en muchos tipos celulares, pero principalmente en células endoteliales, cardiomiocitos y plaquetas. Intracelularmente, eNOS es la única de las tres isoformas que puede estar asociada a membranas, como en las caveolas o el aparato de Golgi. En su estructura se encuentran sitios de unión a FMN, FAD, PKA y CaM, pero lo verdaderamente característico es que posee una molécula de miristoilo, en el extremo N-terminal. Puede ser regulada por miristoilación, transcripción, por modificaciones traduccionales y postraduccionales y por Ca2+, que necesita para ser activa. Controla el flujo sanguíneo en los tejidos y reduce la agregación plaquetaria. La hiperactividad de esta enzima es una de las principales causas de la hipotensión en el shock séptico, y favorece, entre otras patologías, la aterogénesis.

iNOS

Aunque de manera constitutiva se encuentra en pocos tipos celulares y en muy pequeñas concentraciones, puede ser activada en todos los tipos celulares en los que se ha probado. Está asociada a células del sistema inmune. Al igual que las otras isoformas, tiene dominios de unión para NADPH, FMN y FAD, pero es independiente de Ca2+, ya que la CaM se encuentra permanentemente unida a su estructura. Una vez activada, producirá NO hasta que se acaben los sustratos necesarios para su síntesis (MacMicking et al. 1997), por lo que su regulación se produce necesariamente vía transcripción y traducción. Los principales inductores de su activación son endotoxinas o mediadores pro-inflamatorios endógenos. Responde a la endotoxina bacteriana lipopolisacárido (LPS), a las citoquinas interferón γ (IFN-γ) e interleuquina-1B (IL-1β) al TNF-α y a NF-kB. Media los mecanismos de defensa del sistema inmune. Cuando produce NO en grandes cuantías es causante de estados inflamatorios crónicos, como asma, colitis ulcerosa, artritis, y puede favorecer la implantación de ciertos virus o bacterias.

NOS humana	Tamaño cADN	Tamaño proteína	Estructura	Localización subcelular	Presencia	Producc. NO	Cofactores
nNOS	10.0 kb	1433 aa, 161 kDa	homodímero	Citosol >> membrana	Constitutiva	Baja (pM-nM)	BH4, NADPH, FAD, FMN, hemo CaM
eNOS	4.4 kb	1203 aa, 133 kDa	homodímero	membrana >> citosol	Constitutiva	Baja (pM-nM)	BH4, NADPH, FAD, FMN, hemo CaM
iNOS	4.1 kb	1153 aa, 131 kDa	homodímero	Citosol >> membrana	Inducible	Alta (uM)	BH4, NADPH, FAD, FMN, hemo

Tabla 6. Caracterización de las proteínas NOS humanas, adaptación de (Geller & Billiar 1998)

mtNOS

La existencia de esta isoforma ha sido, y es, un tema muy controvertido, siendo que muchos autores no la apoyan. A finales de los años 90 se registró por primera vez la generación de NO en mitocondrias aisladas de hígado de rata (Ghafourifar & Richter 1997; C Giulivi et al. 1998), y rápidamente se observó también en cerebro, corazón, riñón, timo, etc. En años sucesivos se pudo observar que la mtNOS compartía muchas características con la nNOS, y en 2002 el grupo de Giulivi identificó la enzima como un transcrito alternativo de la nNOS, miristoilado y fosforilado (Elfering et al. 2002), asociada a la membrana mitocondrial. Se ha propuesto que la nNOS sigue un proceso de internalización, todavía no bien definido, con participación de proteínas citosólicas como cav-1 (caveolina-1), distrofina, calpaina, de las chaperonas Hsp-90 y Hsp-70 (proteínas de choque térmico 90 y 70, respectivamente), y con modificaciones post-traduccionales como la fosforilación dependiente de Akt en el residuo serina 1412 (Boveris et al. 2010). Yo personalmente comparto esta teoría, y por lo tanto en adelante no trataré la mtNOS como una isoforma independiente.

2. Estructura

Las NOS son enzimas de gran tamaño molecular , entre 135 y 160 kDa (Li & Poulos 2005). Son las únicas enzimas conocidas que necesitan simultáneamente la unión de cinco cofactores: FAD, FMN, el grupo hemo, la tetrahidrobiopterina (BH4), y la Ca2+-CaM. Las NOS necesitan, además, tres co-sustratos para su correcto funcionamiento: L-Arg, oxígeno y NADPH.

Su estructura es compleja; Se sintetizan como proteínas monoméricas, formadas por tres dominios: un dominio oxigenasa y un dominio reductasa, separados entre sí por un dominio de unión a CaM (Conti et al. 2007). El dominio reductasa, en el extremo carboxilo terminal (C-terminal), tiene las zonas de unión a NADPH, FAD y FMN. El dominio de unión a CaM contiene el sitio de unión para Ca2+-CaM. Por su parte, el dominio oxigenasa, en el extremo N-terminal, contiene las zonas de unión a BH4, hemo y arginina. La forma monomérica de la enzima es inactiva, y tiene que formar una estructura homodimérica mediante la unión de dos monómeros en la región hemo del dominio oxigenasa (Li & Poulos 2005). En el homodímero, los dos monómeros interactúan en paralelo, de manera que el dominio oxigenasa de uno de los monómeros está en contacto con el dominio reductasa del otro (Masters et al. 1996). Aunque se suele decir que la forma activa de esta enzima es el homodímero, en realidad es un tetrámero, con dos proteínas homólogas NOS, y otras dos BH4 (Alderton et al. 2001). El dominio reductasa transfiere electrones desde la NADPH hasta el dominio oxigenasa de la otra unidad del dímero. El dominio oxigenasa cataliza la conversión de L-Arg en L-citrulina y NO (Conti et al. 2007).

Las estructuras cristalinas de las tres isoformas muestran que comparten más del 50% de homología. El sitio activo, además, está muy conservado: todos los residuos que tienen contacto directo con el sustrato se han conservado en las tres isoformas (Crane et al. 1998; Raman et al. 1998; Fischmann et al. 1999; Li et al. 2002).

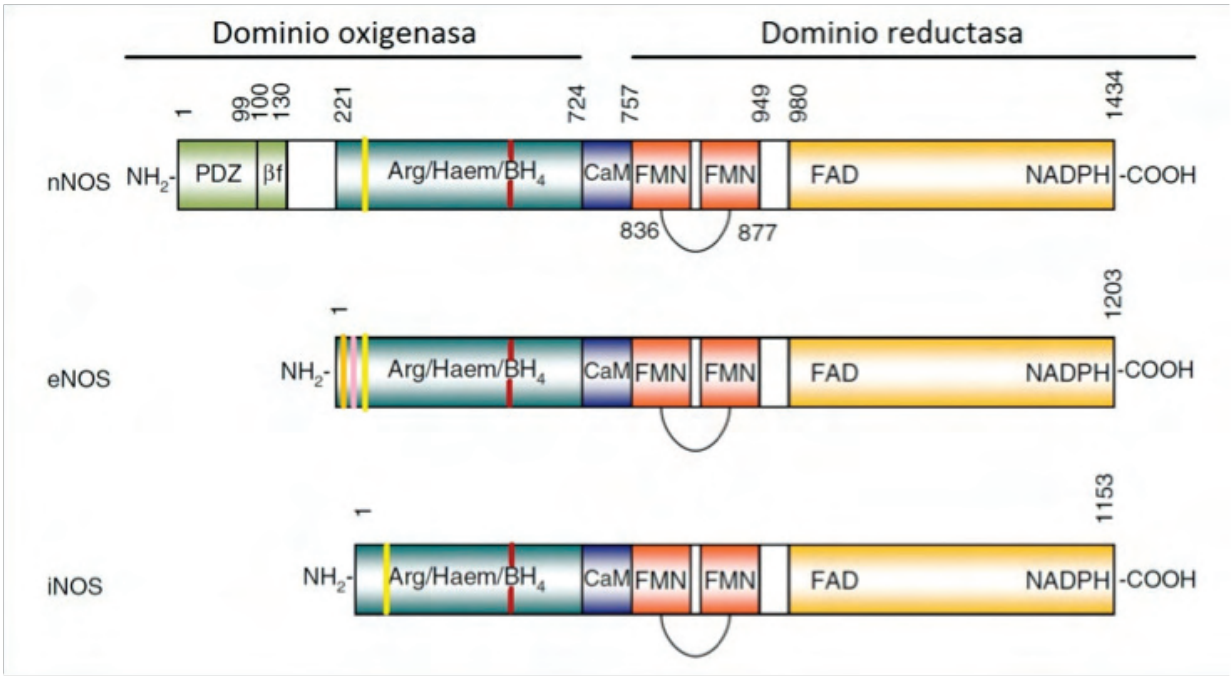


Imagen 23. Comparación de los dominios de las NOS humanas (Alderton et al. 2001; Doucet et al. 2012). Isoformas nNOS (NOS neuronal o NOS-1), eNOS (NOS endotelial o NOS-3) e iNOS (NOS inducible o NOS-2). Los números muestran los residuos de aminoácidos. Se han indicado con líneas el residuo de cisteína de los sitios de unión de los grupos hemo y CaM (marrón), los sitios de miristoilación (naranja), palmitoilación (rosa), las cisteínas de unión a zinc (amarillo) y el bucle de unión autoinhibitoria de las regiones FMN (negro). La dimerización crea zonas de unión (se indican en la imagen) de gran afinidad para la L-Arg (Arg), el grupo hemo (Haem) y la BH4, necesarios para la activación de las NOS (Zhou & Zhu 2009). Las NOS, al igual que el citocromo P450 reductasa, tiene sitios de unión (se muestran en la imagen) para los cofactores NADPH, FAD y FMN (Hobbs et al. 1999). La nNOS es la única que presenta la región PDZ en el dominio oxigenasa.

2.1. Dominio reductasa

Es el extremo C-terminal, y contiene las zonas de unión a NADPH, FAD y FMN. El dominio reductasa de la NOS es similar al del citocromo P450, ampliamente estudiado. Su principal función es permitir que la NADPH (donador de dos electrones) done electrones al grupo hemo (o aceptor de un electrón), formando intermediarios estables de radicales semiquinona (Alderton et al. 2001).

NADPH

Actúa como donador de dos electrones para la activación del oxígeno y la oxidación del sustrato (Ricciardolo et al. 2006).

Cofactores flavinas FAD y FMN

Transfieren electrones desde NADPH (donador de dos electrones) hasta el hierro del grupo hemo (aceptor de un electrón) (Alderton et al. 2001).

A través de estudios de caracterización de mutantes deficientes en FMN, se estableció que el flujo de electrones se da en la dirección NADPH→FAD→FMN→hemo (Adak et al. 1999).

2.2. Zona de unión a Calmodulina

La calmodulina (CaM) transfiere electrones desde las flavinas al grupo hemo y une los dominios reductasa y oxigenasa. Funciona como punto de control en el flujo de electrones entre FAD y FMN, que se ralentiza en ausencia de Ca2+-CaM (Matsuda & Iyanagi 1999). El flujo de electrones se entrecruza entre las dos subunidades de los dímeros, y los electrones que salen de las flavinas de una de las cadenas polipeptídicas pasa al grupo hemo de la otra, lo que podría explicar fisiológicamente por qué los monómeros de la NOS son inactivos (Siddhanta et al. 1996).

La CaM funciona como un activador alostérico de las NOS (Abu-Soud et al. 1994). Se dice que las isoformas constitutivas son dependientes de CaM porque nNOS y eNOS contienen un inserto de 40-50 aminoácidos en la zona de unión de FMN que sirve de giro autoinhibitorio, desestabilizando la unión de CaM a bajas concentraciones de Ca2+ e impidiendo la transferencia de electrones de FMN al grupo hemo cuando no hay Ca2+-CaM (Nishida & de Montellano 1999; Nishida & de Montellano 2001). Este inserto no está en la iNOS (Kone et al. 2003).

2.3. Dominio Oxigenasa

Es el extremo N-terminal, donde se encuentran los sitios de unión del grupo hemo, zinc, BH4 y L-Arg. El dominio oxigenasa cataliza la conversión de L-Arg en L-citrulina y NO.

Hemo

Representa el centro catalítico responsable de la unión y la reducción del oxígeno molecular y la subsecuente oxidación del sustrato (Ricciardolo et al. 2006).

El grupo hemo de las NOS es similar al de la enzima P450. Cataliza la oxidación de las moléculas de substrato cercanas (al propio grupo hemo), aunque no de las unidas directamente. Es esta una oxidación multielectrón, con dos ciclos de oxidación separados: uno para formar N-hidroxiarginina (NHA) (catalizado por la monoxigenasa I), y el otro (catalizado por la monoxigenasa II) para convertir NHA en NO (o NO–) (Alderton et al. 2001). El grupo hemo también participa en la dimerización creando enlaces tipo van der Waals con los aminoácidos vecinos (Kone et al. 2003).

Zinc

El dominio oxigenasa presenta una zona de unión a zinc, muy conservada en todas las isoformas, en el que participan dos cisteínas de cada subunidad. Las cisteínas están colocadas de tal manera que favorecen las interacciones del extremo N-terminal de la propia subunidad. El zinc se coordina con las dos cisteínas de cada subunidad, formando ocho enlaces tipo puentes de hidrógeno, esenciales para la estabilidad del dímero (Chen et al. 1995). Esto favorece la unión de BH4, pero no afecta a la actividad (Alderton et al. 2001).

BH4

La BH4 regula la transferencia de electrones y estabiliza el homodímero de NOS (Panda et al. 2002). BH4 se une al dominio oxigenasa, interaccionando con el grupo hemo, permitiendo la actividad de la NOS (Knowles & Moncada 1994).

Además, se le han asignado otras funciones: promueve el acoplamiento entre la oxidación de NADPH y la síntesis de NO (inhibiendo la formación de superóxido y peróxido de hidrógeno); favorece la formación del dímero y lo estabiliza; Tiene efectos alostéricos en la subsecuente unión de otra molécula de BH4 y la de L-Arg; modifica el entorno del grupo hemo y protege de la inactivación y autoinactivación (Alderton et al. 2001).

L-Arginina

La disponibilidad de L-Arg es indispensable para la formación de NO. La concentración de L-Arg en el líquido extracelular es muy superior al punto de saturación de la enzima. Al ser una aminoácido que se consume en la dieta, su deficiencia se puede suplir fácilmente por administración oral (Bruckdorfer 2005).

2.4. Dímeros de NOS

A pesar de su similitud estructural, los homodímeros de las tres isoformas de la NOS son muy diferentes en la fuerza de la asociación de los monómeros, su interfaz, y la influencia de la L-Arg y la BH4 en su formación y estabilidad, dejando una puerta abierta a su regulación (Kone et al. 2003). La dimerización depende de la unión de L-Arg, hemo y BH4 (Stuehr 1997). L-Arg y BH4 favorecen la formación del dímero de nNOS e iNOS y lo protegen frente a la proteólisis de tripsinas, pero la eNOS es resistente a la proteólisis en cualquier circunstancia. La dimerización, a su vez, potencia la actividad de las NOS al crear uniones de gran afinidad para L-Arg y BH4, facilitando el flujo de electrones desde el dominio reductasa al oxigenasa. La estabilidad de los dímeros parece seguir este orden: eNOS > nNOS > iNOS (Venema et al. 1997).

Cada subunidad de las NOS consiste en un dominio oxigenasa y uno reductasa. Los electrodos donados por la NADPH se transfieren del dominio reductasa al dominio oxigenasa vía FAD, FMN y CaM, oxidando la L-Arg para formar L-citrulina y NO. En presencia de Ca2+, la CaM forma un complejo Ca2+-CaM, permitiendo el flujo de electrones. Los monómeros no pueden unir el cofactor BH4 ni la L-Arg, y por lo tanto no pueden catalizar la producción de NO. La presencia del grupo hemo permite la dimerización de la NOS.

3. Actividad de las NOS: síntesis de NO

Inmediatamente después de identificar el NO, el grupo de Moncada demostró que éste se sintetizaba a partir del aminoácido L-Arg (Palmer et al. 1988), que se encuentra presente de manera natural en altas concentraciones (60-80 uM) en la sangre y el fluido extracelular, y en concentraciones todavía mayores en el interior de las células (Bruckdorfer 2005). La síntesis de NO es una reacción de oxidación de cinco electrones, catalizada por las enzimas NOS.

Como se ha visto en el apartado anterior, la síntesis de NO depende de la presencia de sustrato suficiente y de los cofactores NADPH, BH4, FAD, FMN, oxígeno y protoporfirina IX (hemo). Como productos de la reacción se obtienen NO, NADP+ y L-citrulina, aunque hay que destacar que las NOS también son capaces de producir anión superóxido y peróxido de hidrógeno (Vásquez-Vivar et al. 2003). En líneas generales, por cada mol de NO formado, se consumen dos moles de oxígeno y 1,5 moles of NADPH (Higgins & Gross 2010).

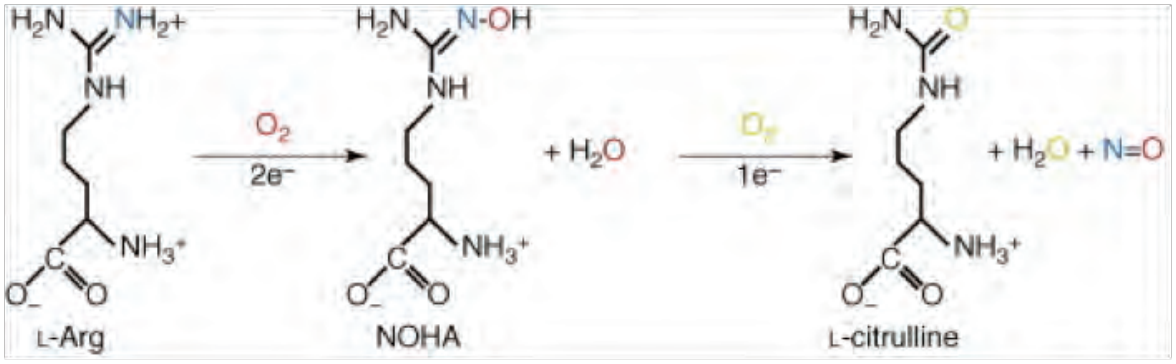


Imagen 24. Biosíntesis de NO a partir de L-Arg (Sudhamsu & Brian R Crane 2009)

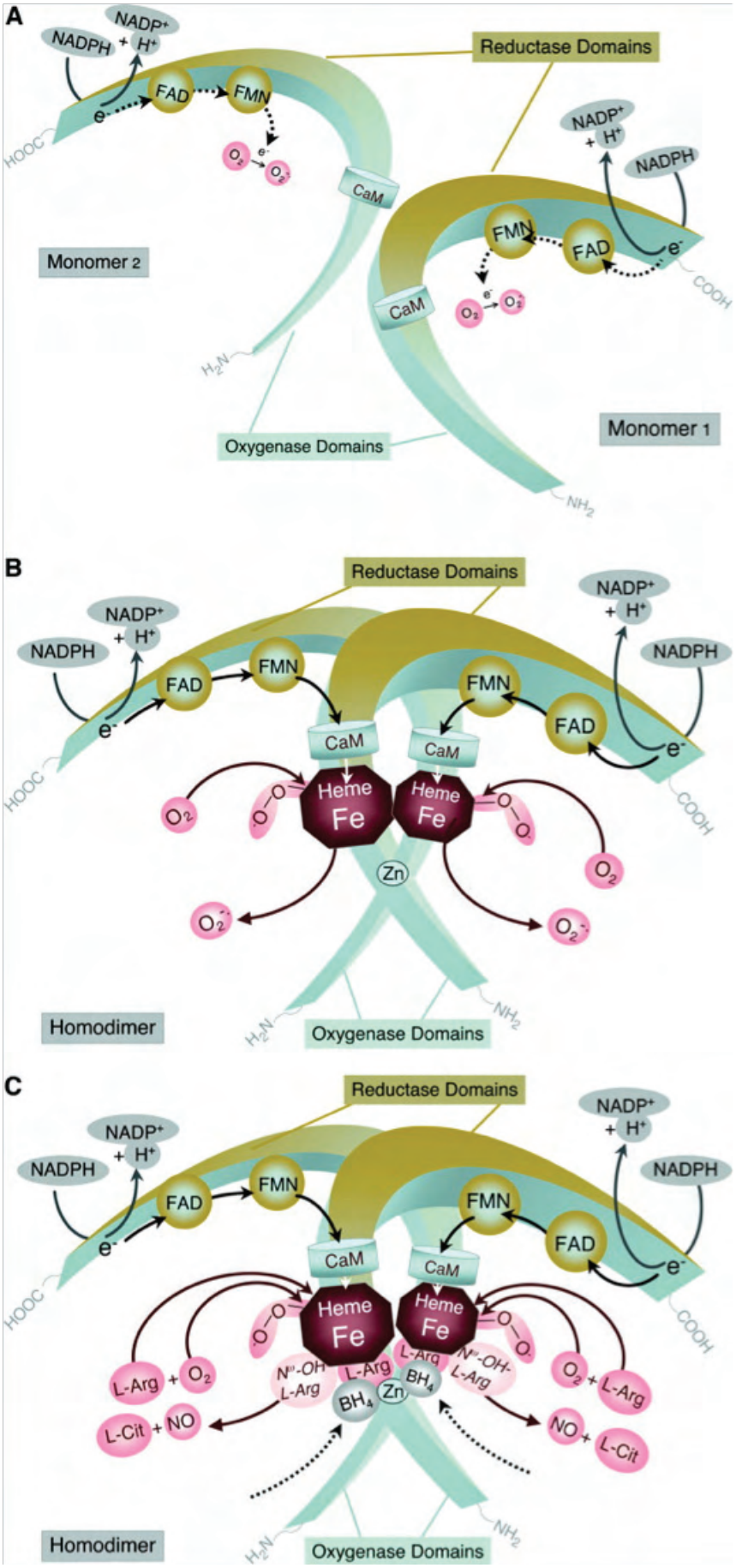

A
NADPH
NADP+ + H+
FAD
FMN
e-
HOOC
Reductase Domains
CaM
Monomer 2
Monomer 1
Oxygenase Domains
H2N
NH2
COOH
B
Heme Fe
Zn
Homodimer
C
L-Arg + O2
L-Cit + NO
BH4
N-OH-L-Arg

Imagen 25. Mecanismo de síntesis de NO por las NOS (Förstermann & Münzel 2006). (A) Todas las enzimas NOS se sintetizan como monómeros. Cada subunidad consiste en un dominio oxigenasa y un dominio reductasa. Los monómeros, y los dominios reductasa por si solos son capaces de transferir los electrones de NADPH a las flavinas FAD y FMN y tienen una capacidad limitada para reducir el oxígeno molecular a O2–. Los monómeros y los dominios reductasa aislados pueden unir CaM, lo que estimula la transferencia de electrones al dominio reductasa. Sin embargo, los monómeros no pueden unir el cofactor BH4 ni la L-Arg, y por lo tanto no pueden catalizar la producción de NO. (B) La presencia del grupo hemo permite la dimerización de la NOS. El grupo hemo es el único cofactor indispensable para la interacción entre los dominios reductasa y oxigenasa y para la transferencia de electrones de un dominio a otro, desde las flavinas al grupo hemo del monómero opuesto. En ausencia de sustrato, las enzimas con el grupo hemo oxidan NADPH, produciendo O2–. (C) Los electrodos donados por la NADPH se transfieren del dominio reductasa al dominio oxigenasa vía FAD, FMN y CaM (Alderton et al. 2001; Zhou & Zhu 2009). El sitio de unión a CaM regula el flujo de electrones entre las dos regiones (Su et al. 1995; Hobbs et al. 1999). En presencia de Ca2+, la CaM se une a Ca2+ para formar un complejo Ca2+-CaM (D S Bredt & S H Snyder 1990; Su et al. 1995), permitiendo el flujo de electrones y la oxidación de la L-Arg para formar L-citrulina y NO. En presencia de cantidades suficientes de sustrato (L-Arg) y cofactor BH4, los dímeros de NOS acoplan la reducción de O2 para sintetizar NO y L-citrulina.

4. Expresión de las NOS

nNOS

Se suele decir que la nNOS es muy abundante en el cerebro (Chabrier et al. 1999); en realidad las neuronas positivas a nNOS representan aproximadamente el 1% de los somas de la corteza cerebral, pero están tan ramificadas que virtualmente todas las neuronas de la corteza cerebral están expuestas a terminaciones nerviosas de nNOS (Barañano et al. 2001), y ésta se encuentra tanto en neuronas presinápticas como en neuronas post-sinápticas (Esplugues 2002). En el SNC, la nNOS está en neuronas y nervios perivasculares, y en menor medida en astrocitos y vasos sanguíneos (Conti et al. 2007). Se observa en la corteza cerebral, el núcleo endopiriforme ventral, el claustrum, el bulbo olfatorio, el núcleo olfatorio, el núcleo accumbens, el estriado, la amígdala, el hipocampo, el hipotálamo, el tálamo, el núcleo peduculopontineo tegmental, el núcleo dorsolateral, el cuerpo trapezoide, el rafé magno, el núcleo del tracto solitario y el cerebelo. En el SNP también se ha observado en neuronas no-colinérgicas, no-adrenérgicas que inervan el músculo liso del tracto gastrointestinal, en los cuerpos cavernosos del pene, en la uretra y la próstata (Calabrese et al. 2007), en dominios post sinápticos de la unión neuromuscular, en el músculo esquelético (Yang et al. 1997);

También se ha encontrado la nNOS en cardiomiocitos (Brahmajothi & Campbell 1999), en células de la mácula densa del riñón; en células epiteliales del pulmón; en mastocitos en la piel; en neutrófilos (Förstermann et al. 1998) y, por supuesto, en células cromafines.

No se conoce ninguna célula humana que no sea capaz de sintetizar NO, lo que nos da una idea de la importancia y variedad de localizaciones donde se expresan las NOS. Dos de las tres isoformas, la nNOS y la eNOS, se expresan de manera constitutiva en los tejidos. La nNOS se encuentra principalmente en células de tipo neuronal, y en miocitos. La eNOS se expresa principalmente en células endoteliales. La iNOS "no" se expresa de manera constitutiva, pero se puede inducir su expresión en prácticamente todas las células. En el sistema nervioso se han encontrado las tres isoformas de la NOS.

eNOS

La eNOS se identificó por primera vez en las células endoteliales (Förstermann et al. 1991; Pollock et al. 1991) del tejido arterial y venoso. En el SNC regula el flujo sanguíneo cerebral, aunque también se ha demostrado su presencia en células no endoteliales, en pequeñas poblaciones de neuronas (Chabrier et al. 1999) piramidales en el hipocampo, en células granuladas del giro dentado, en astrocitos (Calabrese et al. 2007) y motoneuronas (Förstermann et al. 1998). En la periferia se encontrado en el endotelio vascular y en el músculo liso de los cuerpos cavernosos (Calabrese et al. 2007).

Se expresa principalmente en células endoteliales, cardiomiocitos y plaquetas, donde sirve como el primer mecanismo de defensa ante el desarrollo de enfermedades vasculares (Geller & Billiar 1998). También se ha demostrado su expresión en fibroblastos, osteoblastos, queratinocitos, células epiteliales de las glándulas endometriales, megacariocitos, células T, monocitos, hepatocitos, en la mucosa gastrointestinal... (Förstermann et al. 1998; Brahmajothi & Campbell 1999).

iNOS

La iNOS no se encuentra en tejidos sanos, aunque en miocitos se ha visto que se expresa de manera constitutiva en muy bajas concentraciones (Geller & Billiar 1998; Brahmajothi & Campbell 1999). En condiciones patológicas como inflamación, trauma, o como parte de la respuesta inmune, se induce su expresión en macrófagos, astrocitos, microglía, vasculatura del músculo liso, neuronas, células endoteliales, mastocitos, linfocitos, hepatocitos, cardiomiocitos, músculo esquelético... (Chabrier et al. 1999; Conti et al. 2007; Rodrigo et al. 2002), virtualmente, todos los tipos celulares en los que se ha probado (Blaise et al. 2005; Conti et al. 2007). La inducción de la iNOS se produce a nivel transcripcional, mediada por citoquinas inflamatorias como el IFN-γ, el TNF-α y la IL-1β, que parecen actuar por diferentes rutas de señalización (Conti et al. 2007).

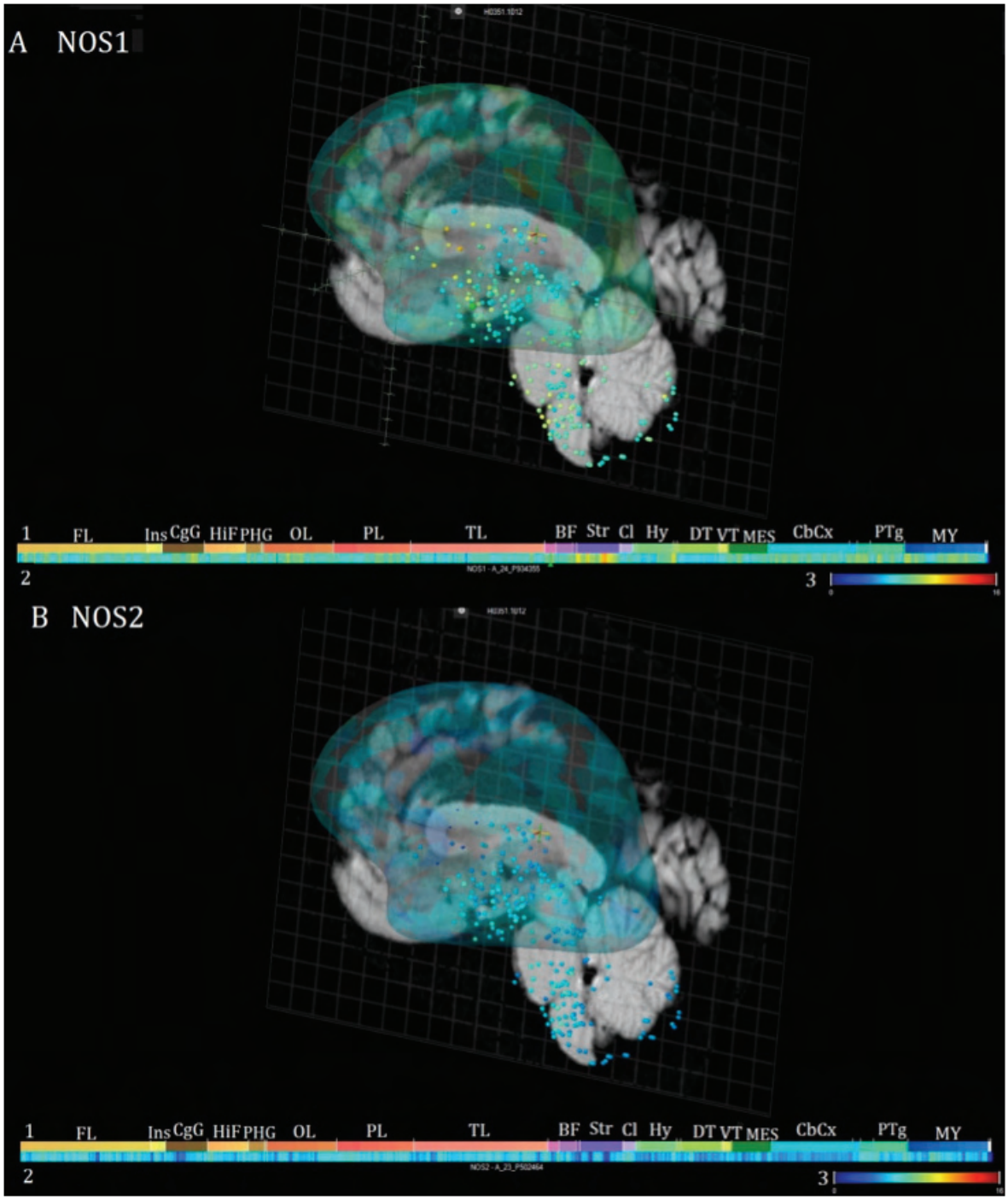


Imagen 26. Niveles de expresión de los genes de nNOS y eNOS en el cerebro humano. La imagen muestra la representación en 3D de los genes nNOS **(A)** y eNOS **(B)** humanos. No se ha representado la iNOS, pues se ha visto que la expresión proteica se da en todos los tipos celulares sometidos a la estimulación precisa (Conti et al. 2007). Las muestras de la corteza cerebral se muestran como volúmenes hinchados superpuestos sobre la superficie de la sustancia blanca, y las zonas de las regiones subcorticales del cerebro se representan como esferas por detrás de estos volúmenes. **(1)** Los colores sólo indican zonas del cerebro, y corresponden a: FL: lóbulo frontal; Ins: ínsula; CgG: giro cingulado; HiF: hipocampo; PHG: giro parahipocampal; OL: lóbulo occipital; TL: lóbulo temporal; BF: prosencéfalo basal; Str: estriado; Cl: claustro; Hy: hipotálamo; DT: tálamo dorsal; VT: tálamo ventral; MES: mesencéfalo; CbCx: corteza cerebelar; PTg: tegmento pontino y MY: mielencéfalo. **(2)** Los colores representan la expresión relativa en las estructuras del cerebro de **(1)**. La escala de color está indicada en **(3)**, donde el azul oscuro es mínima expresión y el rojo, máxima. Estos datos están tomados del cerebro de un varón blanco, de 31 años, en buen estado de salud (antes de morir, claro). Las imágenes se desarrollaron con el excelente programa Allen Human Brain Atlas, del Allen Institute, disponible en http://human.brain-map.org/ (Hawrylycz et al. 2012).

5. Localización subcelular

Las moléculas de NOS no se encuentran distribuidas de forma aleatoria por el citosol, sino que están localizadas en distintas estructuras subcelulares. Esta localización específica es crítica para que la producción de NO se produzca de manera tal que sea capaz de acoplarse a las señales intra y extracelulares adecuadas. Pero, ¿hasta qué punto la localización intracelular de las NOS es capaz de regular la actividad de las NOS?

Cada una de las tres isoformas de la NOS se establece en diversas localizaciones intracelulares mediante diferentes mecanismos. La isoforma eNOS se encuentra anclada a membranas lipídicas; nNOS e iNOS no están anclados a las membranas, pero sí hay regiones en las células más ricas en estas proteínas (Govers & Oess 2004). Los mecanismos más utilizados para regular la localización subcelular de estas proteínas son las modificaciones lipídicas (miristoilación y palmitoilación) y las interacciones tipo proteína-proteína, aunque también se puede regular por fosforilación (Oess et al. 2006). La miristoilación es una modificación proteica co- o post-traduccional irreversible, consistente en la unión covalente de un grupo miristoilo al grupo amino de un aminoácido del residuo N-terminal; es frecuente en los residuos de glicina. La palmitoilación es una modificación proteica post-traduccional reversible, consistente en la adición de ácidos grasos a residuos de cisteínas, serinas o treoninas, típicos de proteínas de membrana. Normalmente la miristoilación es un paso previo necesario para que se produzca la palmitoilación.

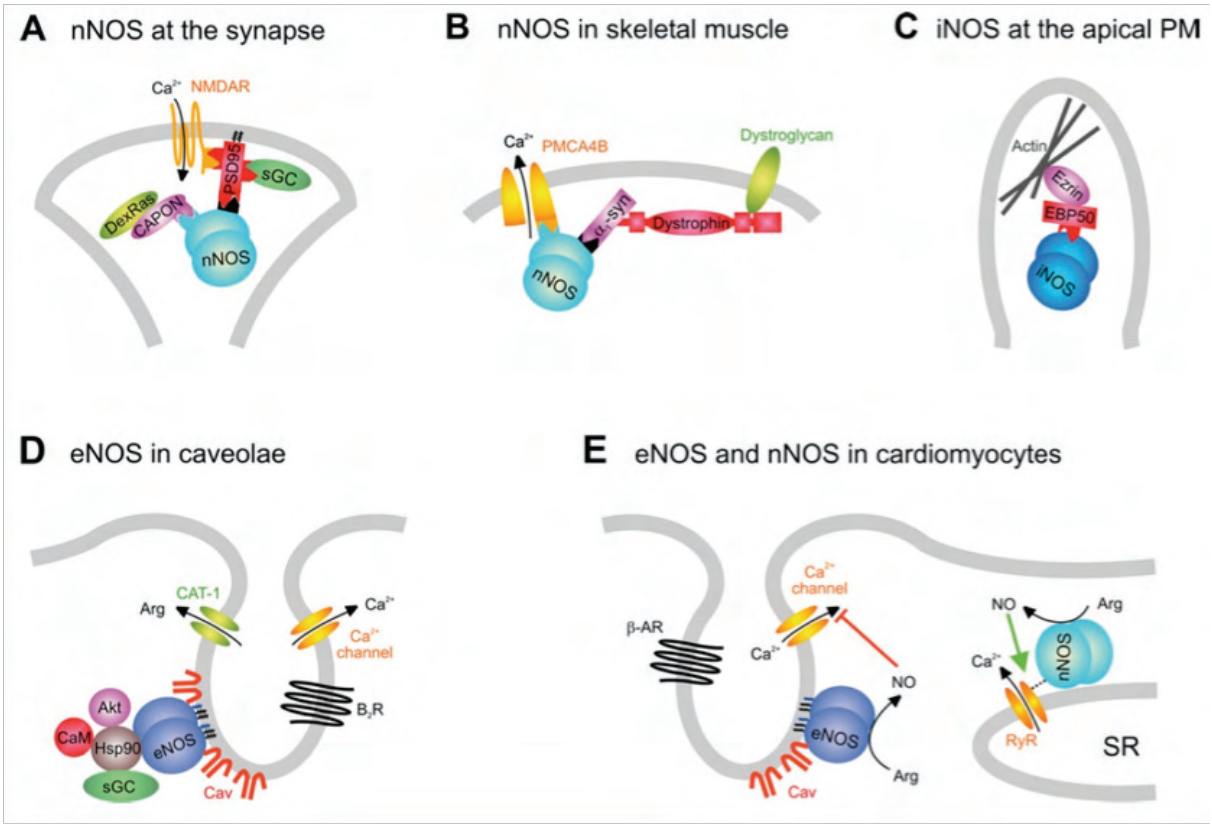


Imagen 27. Asociación de las NOS con membranas biológicas (Oess et al. 2006). (A, B) nNOS en (A) la sinapsis y en (B) el sarcolema del músculo esquelético. (C) iNOS en la membrana apical. (D) eNOS en las caveolas de la membrana endotelial. (E) Distribución diferencial de eNOS y nNOS en la membrana de cardiomiocitos. α1-Syn: α1-sintrofina; β-AR: receptor β-adrenérgico; B2R: receptor B2 de bradiquinina; CAT: transportador de aminoácidos catiónicos; RyR: receptor-1 de rianodina; SR: retículo sarcoplásmico.

5.1. Localización subcelular de nNOS

La localización subcelular de la nNOS varía según el tipo celular en el que se encuentre. Se expresa en neuronas maduras e inmaduras (Zhou & Zhu 2009), y se puede localizar tanto en citosol como asociada a la membrana plasmática (Ricciardolo et al. 2006). La fracción asociada a membrana puede suponer entre un 30 y un 60% de la proteína total; este porcentaje depende en cierta medida del estado de maduración de la célula (Zhou & Zhu 2009). En el músculo esquelético se encuentra principalmente en la fracción particulada (Förstermann et al. 1998).

Interacciones tipo proteína-proteína

La nNOS utiliza para su anclaje a membrana interacciones tipo proteína-proteína. En el extremo N-terminal de la nNOS se encuentra una secuencia de 220 aminoácidos que contienen un dominio de homología PDZ. La nNOS es la única de las tres isoformas de la NOS que posee esta estructura (Govers & Oess 2004).

Los dominios PDZ son pequeños interfaces que modulan interacciones proteína-proteína, y juegan un papel importantísimo en la unión a receptores, canales, efectores en complejos proteicos en uniones celulares y en la interacción con otras proteínas asociadas a la membrana (Ponting et al. 1997; Schepens et al. 1997). El dominio PDZ posee dos motivos: una secuencia consenso y una secuencia "pseudo-péptido" (Wang et al. 2000), que permiten su unión a otras moléculas adaptadoras, como PSD-93, PSD-95 o CAPON (protein carboxy-terminal PDZ ligand of nNOS) (Jaffrey et al. 2002) en neuronas, pero también a la α1-Syn en miocitos (Christopherson et al. 1999; Hillier et al. 1999). La interacción entre el dominio PDZ y PSD-95 es determinante en las nNOS post-sinápticas, pues une la proteína nNOS con el NMDAR, y explica la activación de nNOS cuando se estimula el NMDAR. CAPON es una proteína adaptadora que contiene el dominio PDZ; CAPON interacciona con DexRas1, un miembro de la familia Ras, que se induce selectivamente por dexametasona. La interacción entre CAPON y nNOS permite la S-nitrosilación de Dexras1 (Zhou & Zhu 2009).

Modificaciones lipídicas, miristoilación y palmitoilación

La nNOS, a diferencia de las otras isoformas, no se acetila, y usa mecanismos alternativos para su localización intracelular específica (Mungrue & Bredt 2004). Sin embargo, la proteína PSD-95, que sirve como nexo para la unión al NMDAR (Kim & Sheng 2004), se une a la membrana post-sináptica por palmitoilación (Imamura et al. 2002).de la NOS que posee esta estructura (Govers & Oess 2004).

5.2. Localización subcelular de eNOS

La localización subcelular de la eNOS es fundamental para la actividad de la enzima, ya que las enzimas deslocalizadas reducen su actividad (Sessa et al. 1995). Los contactos célula-célula son zonas muy ricas en presencia de eNOS (Govers et al. 2002); intracelularmente se localiza en la membrana plasmática, en las caveolas (Geller & Billiar 1998), en el complejo de Golgi y se ha observado una pequeña porción de la eNOS en el citosol (Robinson et al. 1995), pero no se sabe si esta última está activa o si es activable (Geller & Billiar 1998). La eNOS transloca de la caveola al núcleo tras la activación con VEGF (McNaughton et al. 2002).

La unión a membranas de la eNOS es un proceso complejo que depende de la palmitoilación, la miristoilación y la fosforilación en tirosina de las enzimas, así como interacciones tipo proteína-proteína con las caveolinas (García-Cardeña et al. 1996; Feron et al. 1996).

Interacciones tipo proteína-proteína
Similar a la situación de la nNOS con PSD, eNOS se encuentra en las caveolas, unida a las proteínas caveolina. También se ha asociado con el citoesqueleto y los contactos célula-célula, con la actina, mitocondria y el núcleo (Oess et al. 2006).

Modificaciones lipídicas, miristoilación y palmitoilación
La eNOS está asociada la membrana plasmática y al aparato de Golgi. El anclaje a estas membranas se produce a través de un proceso de dos etapas: la primera, la miristoilación de un residuo de glicina (Gly-2) en el dominio N- terminal, que produce una asociación a membranas en general. La segunda etapa es la palmitoilación dual de dos residuos de cisteína localizados en el extremo N-terminal (Cys-15 y Cys-26). La palmitoilación permite la unión de la eNOS a la membrana plasmática y/o las caveolas. Este proceso facilita también la interacción de eNOS con la caveolina, y la unión a las caveolas de la membrana plasmática. La despalmitoilación inicia el transporte retrogrado desde la membrana plasmática hasta el aparato de Golgi. Las eNOS miristoiladas no palmitoiladas son menos activas (Oess et al. 2006).

5.3. La localización subcelular de la iNOS

La iNOS no está anclada a la bicapa lipídica, pero su presencia está enriquecida en ciertas zonas de la célula (Geller & Billiar 1998). Por ejemplo, en macrófagos se encuentra en ciertas fracciones de membrana (Förstermann et al. 1992); en el músculo esquelético se expresa de manera constitutiva en muy bajas concentraciones, y la mayor parte de la enzima se encuentra en la membrana, asociada a la Cav-3 (García-Cardeña et al. 1997); en las células epiteliales iNOS se encuentra unida al dominio apical en un complejo proteico submembrana fuertemente unido a actina (Glynne et al. 2002).

Interacciones tipo proteína-proteína
De manera similar al mecanismo utilizado por la nNOS, y aunque no posea el motivo PDZ, la iNOS es capaz de unirse a la proteína EBP50 (ezrin/radixin/moesin-binding phosphoprotein-50), que sí contiene un dominio PDZ, y localizarse en el dominio apical de las células epiteliales (Oess et al. 2006).

Modificaciones lipídicas, miristoilación y palmitoilación
Una parte de la fracción total de la iNOS puede ser palmitoilada, aunque no miristoilada, y anclada a la membrana plasmática –pero no específicamente a las caveolas-, y parece ser necesaria para la producción de NO. Por otra parte, la localización de iNOS en las caveolas disminuye la estabilidad de la enzima, contribuyendo a una disminución en la producción de NO (Oess et al. 2006).

6. Regulación de la NOS

La regulación de la NOS puede llevarse a cabo de muchas maneras, como reseñamos a continuación. En la tabla 1 se resume la regulación de las diferentes isoformas, y en la tabla 2 se muestran las principales proteínas que interaccionan con las NOS. Para un estudio más profundo se recomiendan las revisiones de (Kone et al. 2003; Bolaños et al. 1997; Alderton et al. 2001; Nicholls 2004).

Regulación de las NOS

Tipo de Regulación	Molécula	nNOS	eNOS	iNOS
		Actividad		
[Sustrato]	L-arginina	Necesario	Necesario	Necesario
[Co - sustrato]	Oxígeno	Necesario	Necesario	Necesario
[Co - sustrato]	NADPH	Necesario	Necesario	Necesario
[Co - factor]	FAD	Necesario	Necesario	Necesario
[Co - factor]	FMN	Necesario	Necesario	Necesario
[Co - factor]	hemo	Necesario	Necesario	Necesario
[Co - factor]	BH_4	Necesario	Necesario	Necesario
Interacciones proteína-proteína	CaM	Sí (↓)	Sí (↓)	No
Producto	NO	(↓)	(↓)	(↓)
Interacciones proteína-proteína	PIN	Sí (↓)		
Interacciones proteína-proteína	Hsp90	Sí (↑)	Sí	Sí (↑)
Interacciones proteína-proteína	Kalirina		Sí (↑)	Sí
Modificaciones covalentes	Fosforilación	Sí (↓) PKA, PKC, CaMKI, CaMKII	PKB/Akt, PI3K/Akt	Sí CaMKII
		Localización subcelular		
Modificación lipídica	Miristoilación	No	Sí	No
Modificación lipídica	Palmitoilación	No	Sí	Sí
Modificación covalente	Fosforilación	Sí	Sí	Sí
Interacciones proteína-proteína	NOSIP		(↓)	
Interacciones proteína-proteína	Caveolina	(↓)	(↓)	
Interacciones proteína-proteína	Dominios PDZ			
		Regulación de la expresión		
Factores de transcipción	NF-κB STAT - 1ª STAT3, NR, ER			Sí
Estabilidad del mARN				Sí
Splicing alternativo		Sí	¿ ?	Sí

Tabla 7. Esquema de la regulación de las NOS

Proteínas que interactúan con las NOS, regulándolas

	nNOS	eNOS	iNOS
Activadores alostéricos	CaM Hsp90	CaM Dinamina-2	CaM Rac-2
Inhibidores	PIN NOSIP	Cav-1 Dominio ID4	Kalirina NAP110
Receptores	B2-R a1-A-R	B2R ET-1 ETB-R AT1-R a1-A-R 5-HT2B-R	a1-AR
Adaptadores / soportes	Cav-3	Hsp90 Cav-3 NOSIP NOSTRIN	EBP-50 -1 Cav
Transportadores	PMCA 4b	Porina CAT-1	
Otros dominios PDZ	PSD-95, PSD-93 α1-Syn CAPON PFK-M		

Tabla 8. Proteínas que interaccionan con las NOS, regulándolas (Kone et al. 2003) PIN: proteína inhibidora de NOS; α1-AR: α1A-Adrenergic receptors: PFK-M: fosfofructoquinasa M; NOSIP: proteína que interacciona con las NOS

6.1. Regulación de la actividad

Las isoformas nNOS y eNOS regulan de muchas maneras su actividad, a diferencia de la iNOS, que se regula principalmente a nivel de expresión por mecanismos transcripcionales y post-transcripcionales. De todos modos, se puede observar que la actividad de la iNOS se puede ver regulada, como la de las cNOS (NOS constitutivas), por la disponibilidad de arginina, o factores que impidan la formación del dímero, o por ciertas proteínas.

6.1.1. Dimerización

La dimerización aumenta la actividad de las NOS al crear sitios de unión de gran afinidad por la BH4 y la L-Arg, facilitando el flujo de electrones. Como se ha comentado previamente, el flujo de electrones pasa de un monómero a otro, por lo que los monómeros de la NOS son inactivos. Además, la estabilización de los dímeros protege a las NOS de la proteólisis. La desestabilización del dímero de nNOS hace que sea más susceptible a la fosforilación por PKC y a la hidrólisis por tripsina (Zhou & Zhu 2009).

Proteína inhibidora de NOS
PIN es una molécula compuesta por una cadena larga de miosina y dineina. Inhibe la actividad de la nNOS al unirse a su extremo N-terminal, desestabilizando el dímero nNOS sin alterar su localización (Govers & Oess 2004).

Kalirina
Inhibe la actividad de la iNOS en el cerebro al impedir que se formen los dímeros de la proteína, ejerciendo una función citoprotectora (Ratovitski et al. 1999).

6.1.2. Disponibilidad de co-sustratos y co-factores

Como se mencionó antes, las NOS requieren para su actividad la presencia simultánea de cinco co-factores (FAD, FMN, hemo, BH4 y CaM) y tres co-sustratos (L-Arg, oxígeno y NADPH).

Hemo
La presencia del grupo hemo permite la dimerización de la NOS. El grupo hemo es el único cofactor indispensable para la interacción entre los dominios reductasa y oxigenasa y para la transferencia de electrones de un dominio a otro, desde las flavinas al grupo hemo del monómero opuesto. En ausencia de sustrato, las enzimas con el grupo hemo oxidan NADPH, produciendo O_2^-

Calmodulina
La CaM actúa como un activador alostérico de las NOS. Es necesaria para la actividad catalítica de las tres isoenzimas, ya que es a ella a la que se une el Ca^{2+} y se inicia la transferencia electrónica.

En ausencia de Ca2+-CaM el flujo de electrones de FAD a FMN se ralentiza. Las isoformas constitutivas de la NOS no presentan CaM unida a su estructura. Por su parte, la iNOS está permanentemente unida a la CaM, y este modo, la iNOS siempre está activa, incluso a pequeñas concentraciones de Ca2+ (Govers & Oess 2004).

A concentraciones intracelulares basales de Ca2+, las cNOS están inactivas. Al aumentar la concentración de Ca2+, dos cationes de Ca2+ se unen a la CaM, permitiendo que este complejo Ca2+-CaM se una a las cNOS, activándolas; cuando las concentraciones de Ca2+ vuelven a disminuir, la CaM se disocia de las cNOS y estas se inactivan de nuevo. Es decir, las cNOS están reguladas por la concentración intracelular de Ca2+, que permiten la asociación cNOS-CaM (Zhou & Zhu 2009).

L-arginina

La producción de NO depende de la disponibilidad del sustrato L-Arg (Hibbs et al. 1987; Jorens et al. 1991). En condiciones fisiológicas, la L-Arg no es un limitante. Además, se utiliza el ciclo arginina/citrulina para mantener una concentración adecuada de arginina a partir de citrulina (Kavya et al. 2006). Sin embargo, es interesante destacar que, a pesar de la saturación de las NOS con las concentraciones fisiológicas de L-Arg, éstas siguen dependiendo de L-Arg exógena, lo que se conoce como la "paradoja de la arginina" (Vukosavljevic et al. 2006).

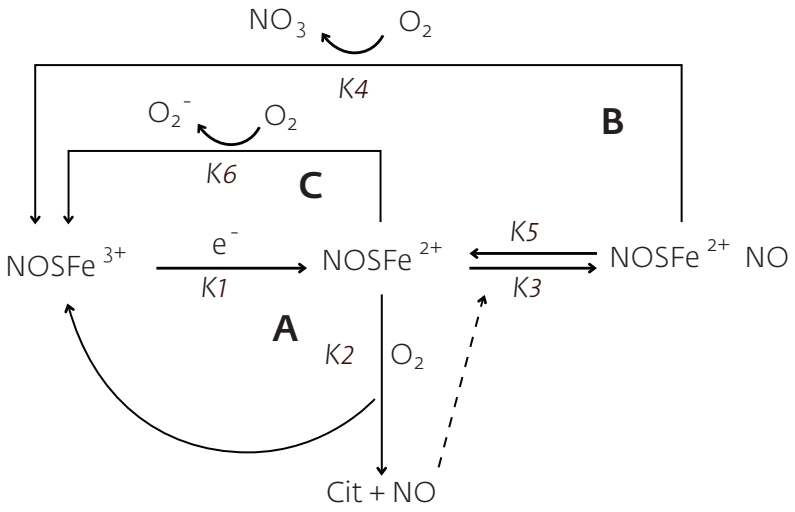


Imagen 28. Inhibición de NOS por NO (Abu-Soud et al. 1995)

6.1.3. Inhibición por NO

El NO inhibe la acción de las NOS al unirse al grupo hemo de la enzima. El NO formado durante la reacción de la NOS compite con el oxígeno por la unión con el hierro (+2) del grupo hemo. Como la síntesis de NO necesita hierro (+2), el complejo Fe-NO vuelve a la NOS catalíticamente inactiva (Abu-Soud et al. 1995).

6.1.4. Fosforilación

Todas las isoformas son susceptibles de fosforilación. La nNOS y la eNOS se fosforilan en serinas por proteínas quinasas.

La fosforilación afecta a la actividad de la nNOS de diferentes maneras. La nNOS tiene tres sitios de fosforilación. En células transfectadas se ha observado la fosforilación del residuo Ser741 por CaMKI. Esta fosforilación disminuye la actividad de la nNOS, pero la expresión de la CAMKI disminuye con el desarrollo del cerebro, y está por demostrar que esto ocurra in vivo. La CaMKII fosforila el residuo Ser847 de la nNOS, reduciendo la actividad de la enzima al impedir la unión de Ca2+ con CaM. La proteína fosfatasa 1 disminuye el nivel de fosforilación de la nNOS en el residuo Ser847, disminuyendo su actividad. Por último, el residuo Ser1412 de la nNOS es análogo al sitio de fosforilación de la Akt de la eNOS. La fosforilación de este sitio aumenta la actividad de la nNOS, y la defosforilación lo disminuye (Zhou & Zhu 2009). La PKC fosforila preferentemente los dímeros de nNOS sin BH_4 (Kavya et al. 2006). La actividad de la eNOS se activa por PKB y PI3K (fosfatidilinositol 3-quinasa). Aunque no se sabe bien de qué modo, también iNOS puede ser regulada por fosforilación, dado que se han encontrado datos donde tiroxinas quinasas y fosfatasas regulan iNOS en macrófagos (Mariotto et al. 2004).

Hsp90
Hsp90 es una chaperona que regula la actividad de la nNOS de manera alostérica. Hsp90 aumenta la unión de CaM a nNOS, activándola. La interacción de Hsp90 con iNOS aumenta su actividad (Yoshida & Xia 2003).

Caveolina-3
Inhibe la unión de Ca2+-CaM, suprimiendo la síntesis de NO en el músculo esquelético (Zhou & Zhu 2009).

6.2. Regulación de la localización subcelular

Como se ha visto, la localización subcelular de las NOS está muy regulada. Oess (Oess et al. 2006) plantea una pregunta muy interesante al respecto: ¿Por qué hay que esforzarse tanto para producir en una localización intracelular determinada de un gas que puede difundir libremente?. Las respuestas son varias.

Por una parte se ha observado que la producción de NO está regulada por la localización de las NOS. Así, se ha planteado que habrá compartimentos que permitan la activación total de la enzima, permitiendo un acceso libre a los sustratos y cofactores, mientras que otros no lo harán, regulando de esta manera la producción de NO. Por ejemplo, la actividad de la iNOS y la producción de NO es invariable tanto en la membrana plasmática, la mitocondria, el aparato de Golgi o el citosol, pero parece ser que en algunos tipos celulares el anclaje de iNOS a la membrana plasmática es necesario para que se produzca NO de manera eficiente (Oess et al. 2006). La actividad de la eNOS varía en función de su localización (ver tabla). Se sabe, por ejemplo, que la NOSIP regula la producción de NO en el sistema nervioso modulando la localización de la eNOS y la nNOS y disminuyendo su actividad (Michel & Vanhoutte 2010). La actividad neuronal puede sobreestimular NOSIP, contrarrestando una producción excesiva de NO y funcionando como mecanismo citoprotector (Zhou & Zhu 2009). La actividad de la eNOS se ha intentado explicar por diferencias en la concentración de Ca^{2+} intracelular: la sensibilidad al Ca^{2+} de la eNOS depende de su estado de fosforilación, y este a su vez depende de la localización intracelular de la eNOS. No se sabe como la localización afecta a la producción de la nNOS, aunque muchos de los sitios de fosforilación de la eNOS (que afectan a su actividad) se conservan en la nNOS (Oess et al. 2006).

Isoforma NOS	Localización subcelular	Actividad
eNOS	Membrana plasmática	Alta
eNOS	Citosol	Baja
eNOS	Golgi (cis)	Moderada
eNOS	Golgi (cis)	Baja
eNOS	Núcleo	Muy baja
eNOS	Mitocondria	Baja
eNOS	Citoesqueleto (actina)	Baja
eNOS	Citoesqueleto (tubulina)	Alta
iNOS	Peroxisomas	Desconocida
iNOS	Membrana plasmática	Alta
iNOS	Núcleo	Alta
iNOS	Mitocondria	Alta

Tabla 9. Producción de NO en compartimentos subcelulares (Oess et al. 2006)

Por otra parte, es importante que haya un acoplamiento eficaz entre la producción de NO con las vías de señalización con las que interacciona, principalmente porque la vida media del NO es muy breve (Geller & Billiar 1998). Por ejemplo, la nNOS está anclada a PSD en la neurona post-sináptica, que a su vez está unida al NMDAR, permitiendo una rápida activación de la nNOS y producción de NO en respuesta a la entrada de Ca2+. Por ello, la expresión de la nNOS es particularmente abundante en las zonas donde se expresa el NMDAR y el NO puede difundir rápidamente a la neurona pre-sináptica. En este ejemplo, el efecto biológico está mediado por activación de sGC, y subsecuente producción de cGMP. La unión de NO a sGC requiere muy pequeñas cantidades de NO, y explica porqué la señal de NO se transmite de una manera tan eficaz a las células vecinas. La localización de NOS en la periferia celular protege a la célula del daño inducido por el NO, importante para células epiteliales, donde iNOS se une a EBP50 produciendo grandísimas cantidades de NO, como defensa ante los patógenos. El NO también actúa de manera independiente a cGMP, pero a mayores concentraciones. La producción de altas concentraciones de NO se da en zonas muy determinadas. Esto se da, por ejemplo, en la nitrosilación reversible de proteínas: la eNOS regula su actividad auto-nitrosilándose; La actividad de la nNOS se puede regular nitrosilando los NMDAR, lo que disminuye la entrada de Ca2+. La proximidad espacial de las NOS con los complejos de señalización celular con los que interactúa es crucial para facilitar la nitrosilación, y permitir mecanismos de tipo feedback, apoyando la idea de que las NOS tienen que estar donde se necesita NO (Oess et al. 2006).

En resumen, podemos concretar que la regulación de la localización subcelular de las NOS se produce bien por interacciones tipo proteína-proteína, bien por modificaciones lipídicas.

6.3. Regulación de la expresión

Todas las isoformas son susceptibles de regular su expresión, aunque este tipo de regulación es mucho más importante en el caso de la iNOS, ya que es el principal mecanismo de regulación que utiliza (Pautz et al. 2010). Así, nNOS y eNOS pueden sufrir cambios en su expresión debido a estímulos como el estrés, la concentración de hormonas sexuales, corticoides, neurotrofinas, neurotransmisores e incluso, regulación por citoquinas y LPS. En la iNOS, las rutas de transducción involucradas en la inducción de su expresión son muy heterogéneas y presentan especificidad de especie y de tipo celular (Kleinert et al. 2004).

6.3.1. Regulación de la expresión de la iNOS

La expresión de la iNOS está regulada por LPS y por citoquinas inflamatorias (IFN-γ, TNF-α, IL-1β), que activan el promotor a través de ciertas regiones reguladoras: NF-κB, secuencia de activación de IFN-γ (GAS), factor nuclear- interleuquina 6 (NF-IL6). Parece ser que la inducción por IFN-γ está mediada por STAT-1 (Signal transducer and activator of transcription-1), mientras que TNF-α, IL-1β utilizan la vía del NF-kB (Conti et al. 2007).

La iNOS sufre regulación transcripcional (NFκB, Jun/Fos, CREB, STAT, citoquinas, LPS, retro-regulación por NO) y post-transcripcional (transforming growth factor TGFB, PKC, cAMP). Como se ha visto antes, las regulaciones traduccionales y post-traduccionales, aunque todavía poco claras, son importantes para controlar la disponibilidad de proteína, y se realizan mediante la estabilización de la proteína, dimerización, fosforilación, unión a cofactores y disponibilidad de sustratos (Aktan 2004).

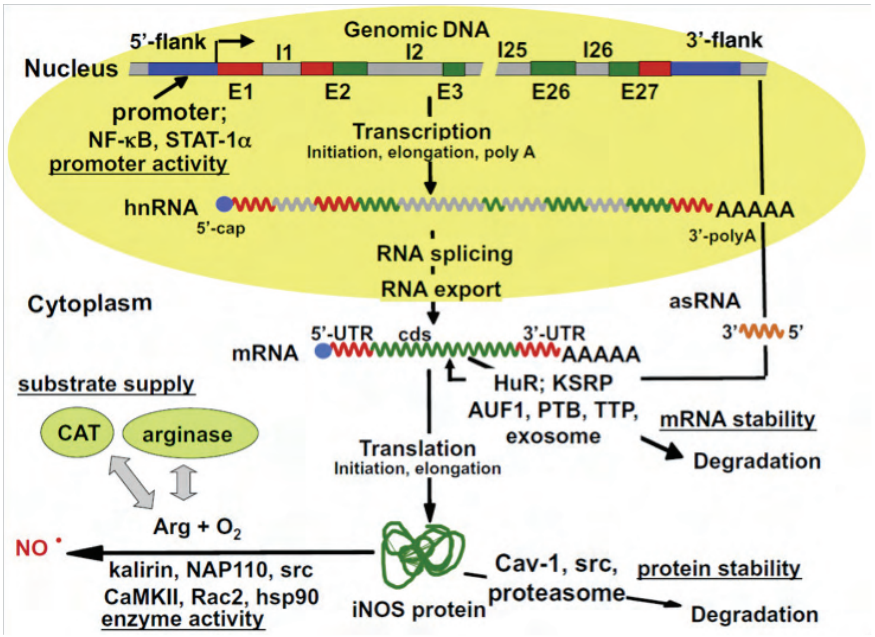


Imagen 29. Niveles de regulación de la expresión de la iNOS y de la producción de NO relacionada (Pautz et al. 2010). La principal forma de regulación de la iNOS es a través de su expresión. Esto incluye la regulación de la actividad del promotor por la unión de factores de transcripción (NF-kB, STAT-1a). Otro mecanismo importante es la modulación de la estabilidad del mARN. La estabilidad del mARN está mediada por la unión de ARN-BP (AUF1, HuR, KSRP, PTB, TTP) al mARN de la iNOS y la interacción con el exosoma. Finalmente, también se puede regular la estabilidad de la proteína iNOS, mediante la interacción con otras proteínas (Cav1, src) y con el proteosoma. La actividad enzimática de la iNOS está regulada por interacciones proteína-proteína (kalirina, NAP110, src, CaMKII, Rac2, hsp90). Como la actividad depende de la presencia del sustrato L-Arg, la iNOS estará regulada por aquellos factores que impidan la presencia del sustrato (proteínas que compiten por el sustrato, o transportadores de membrana que capten L-Arg).

6.3.2. Factores de transcripción en la expresión de la iNOS

La secuencia del promotor (región 5') de todos los genes iNOS de mamíferos presentan muchas homologías en las zonas de unión de varios factores de transcripción, como AP-1, C/EBP, CREB, GATA, HIF, IRF-1, NF-AT, NF-kB, NF-IL6, Oct-1, PARP1, PEA3, p53, Sp1, SRF, STAT-1a y YY1. Los promotores de la iNOS de todas las especies contienen una caja TATA a unas 30pb de la zona de inicio de la transcripción. Cerca de la caja TATA, todos los promotores de mamíferos contienen zonas de unión para NF-kB y por los factores de transcripción inducidos por TNF-a (Pautz et al. 2010).

NF-kB
NF-kB es la principal diana de activadores e inhibidores de la expresión de la iNOS. LPS, IL-1β. TNF-a y el estrés oxidativo inducen la expresión de la iNOS en diferentes tipos celulares, activando NF-kB. Los glucocorticoides, TGF-B y los antioxidantes inhiben la iNOS, al inhibir la activación de NF-kB. Esta inhibición se produce atrapando NF-kB por interacciones tipo proteína-proteína (Kleinert et al. 1996), el bloqueo de la translocación al núcleo de NF-kB (Jeon et al. 1998), la inhibición de la actividad de transactivación de NF-kB (Yu et al. 2002), o el aumento de la expresión de I-kB, inhibidor específico de NF-kB (De Vera et al. 1997; Saura et al. 1998).

STAT-1a, Signal transducer and activator of transcription-1a
Todos los promotores de iNOS de mamíferos contienen varias zonas de unión a STAT-1a. La mutagénesis dirigida del promotor humano de iNOS identificó un dominio bifuncional NF-kb/STAT-1a (Guo et al. 2007). STAT-1a inhibe la función de NF-kB en fibroblastos humanos (Ganster et al. 2001). Además se ha observado la inhibición de la expresión de la iNOS en la ruta JAK-STAT-1ª (JAK, Janus kinases) (Tedeschi et al. 2003). También se ha observado la inducción de iNOS por citoquinas por interacción con c-Fos (Xu et al. 2003).

Activating protein-1 (AP-1)
Se ha observado que inhibidores de las vías de señalización de ERK (Extracellular signal-Regulated Kinase) y p38 MAPK reducen la activación de la iNOS inducida por LPS/ IFN-γ en células A549, disminuyendo la unión de AP-1 con el promotor de la iNOS. Por otra parte, en astrocitos humanos se observó que la inhibición de JNK inhibía la actividad del promotor inducida por IL-1β, pero no la inducida por IL-1β + IFN-γ (Pautz et al. 2010).

STAT-3 (Signal transducer and activator of transcription)/EGF (Epidermal growth factor)
Se ha observado que EGF entra al núcleo y actúa como factor de transcripción. EGF no tienen un dominio de unión a ADN, sino que interactúa con STAT-3, que funciona como cofactor, aumentando la expresión de iNOS.

Receptores nucleares (NR)
Los receptores nucleares son una familia de factores de transcripción inducidos por ligandos. Varios receptores nucleares interaccionan con factores de transcripción pro-inflamatorios como NF-kB y STAT-1a, y reprimen su actividad (Pascual & Glass 2006).

Receptores de glucocorticoides-a, -b (GR-a, -B)
Los glucocorticoides (GC) regulan múltiples procesos bioquímicos (inflamación, apoptosis, crecimiento y desarrollo) (Lu & Cidlowski 2006). Los GC actúan principalmente uniéndose a receptores de glucocorticoides (GR). Los principales efectos antiinflamatorios de los GC se atribuyen a la interacción de los GR con factores de transcripción pro-inflamatorios (NF-kB, STATs). Estas interacciones inhiben la actividad de estos factores de transcripción disminuyendo la expresión de los genes pro-inflamatorios.

Los GC como la dexametasona inhiben la inducción de iNOS en la mayoría de las células analizadas. Los efectos de los GC en la expresión de la iNOS se han atribuido a la inhibición de la actividad de NF-kB mediada por GC, por una interacción directa entre GR y NF-kB (Kleinert et al. 1996), o la inducción de la expresión de I-kB mediada por GC (De Vera et al. 1997). También se ha descrito una regulación post-transcripcional de la expresión de la iNOS mediada por GC mediante la degradación del mARN de la iNOS (Korhonen et al. 2002) o de la proteína iNOS (Walker et al. 1997).

Receptores de estrógenos-a, -b (ER-a, -B)
La transcripción del gen iNOS humano está regulada positivamente por ER-B y negativamente por ER-a en células vasculares del músculo liso. Muchos efectos de los ER están mediados por la interacción de los ER con otros factores de transcripción (Pautz et al. 2010).

Tumor supresor p53
La sobreexpresión de p53 en líneas de células tumorales humanas, ejerciendo una regulación negativa de la expresión de la iNOS por inhibición del promotor de la iNOS. La exposición de células humanas a donadores de NO o la sobreexpresión de iNOS aumentaba la expresión de p53. Por tanto, parece que un mecanismo de retroalimentación negativo parece regular la expresión de la iNOS mediante la expresión de p53 inducida por NO.

Regulación de la estabilidad del mARN de iNOS
Especialmente en el sistema humano, hay grandes diferencias entre la actividad del promotor y la expresión del mARN de la iNOS, por lo que la regulación de la estabilidad del mARN de la iNOS juega un papel fundamental en la inducción de la iNOS (Pautz et al. 2010).

6.3.3. Splicing alternativo

Una forma de regulación de la expresión es a través del splicing alternativo. El splicing alternativo está bien caracterizado en la nNOS, se ha detectado también en la iNOS, pero no en la eNOS, aunque es esta última se han observado mARN de diferentes tamaños. La nNOS está codificada por uno de los genes con mayor diversidad estructural en los humanos. La regulación post-transcripcional del gen de la nNOS se produce por splicing alternativo. Los productos de este splicing se muestran en la Imagen. nNOSa es la proteína conocida como nNOS. En las tablas se muestran las variantes encontradas por splicing.

6.4. Interacción entre las isoformas nNOS-iNOS

En la literatura reciente se empieza a acrecentar la idea de que las isoformas de la NOS no funcionan independientemente, sino que existe entre ellas, bajo ciertas circunstancias, interacciones que participan en su regulación. Mariotto (Mariotto et al. 2004) expone la hipótesis de que la actividad de la isoforma iNOS podría estar regulada por la constitutiva nNOS, a través de NF-kB.

Hasta el momento es bien conocido el hecho de que iNOS se regula a nivel transcripcional por NF-kB. NF-kB se encuentra de manera constitutiva en el citosol celular, formando un complejo inactivo con su inhibidor I-kB. Al estimular la célula con citoquinas o LPS, se produce la fosforilación de I-kB por la proteína quinasa IKK (Inhibitor of KappaB Kinase); I-kB fosforilado se degrada, se rompe el complejo NF-kB-I-kB, y NF-kB se traslada al núcleo e induce la expresión del gen de la iNOS. En contraste, pequeñas cantidades de NO inhiben la activación producida por las citoquinas, impidiendo la activación de iNOS. Esto hizo suponer que el NO producido por la nNOS de forma constitutiva podría ser suficiente para mantener NF-kB en el núcleo, inhibiendo iNOS, y llevó a la hipótesis de que las citoquinas podrían producir una rápida inducción de nNOS. Citoquinas e interferones podrían fosforilar nNOS, inactivándola, y disminuyendo la concentración de NO. Las mínimas concentraciones de NO que ahora se encuentren en las células no serían suficientes para mantener NF-kB inactivado, promoviendo la actividad de iNOS (Aktan 2004; Ashe & Berry 2003; Mariotto et al. 2004).

7. Inhibidores de las NOS

En condiciones patológicas se producen grandes cantidades de NO, que dañan los tejidos y producen estrés oxidativo. Estas altas concentraciones de NO están relacionadas con enfermedades como la artritis reumatoide, el Alzheimer o el Parkinson. Tratándose de otra molécula, la aproximación más sencilla sería diseñar compuestos que redujeran la concentración de la sustancia tóxica. En el caso del NO esta aproximación no es tan sencilla. Impedir que el NO actúe como segundo mensajero, llegando hasta sus dianas fisiológicas, es perjudicial para el organismo. Como el NO se produce principalmente por las NOS, una manera de regular estas concentraciones patológicas sería inhibiendo de manera selectiva las diferentes isoformas de la NOS. Experimentos con ratones transgénicos demostraron que la pérdida de cada una de las isoformas produce el esperado efecto de disminución en la concentración de NO en las respectivas células (Silverman 2010).

Desde finales de los años 80 las compañías farmacéuticas iniciaron programas para identificar inhibidores específicos de la nNOS, intentando buscar tratamiento a las enfermedades neurodegenerativas. Como entonces no se disponía de estructuras cristalinas, la primera aproximación fue utilizar análogos del sustrato L-Arg. Desde entonces se han publicado cientos de artículos. En ellos se presentan muy diversos inhibidores, sin un criterio homogéneo de selectividad frente a las diversas isoformas, que se define con respecto a diferentes parámetros, como pueden ser su IC50, Ki, o ratio, y con distintas medidas (en enzimas purificadas, sobre tejidos, in vivo...). La selectividad de los inhibidores depende también del tipo celular y del tiempo de exposición al inhibidor, así como de la presencia de los cofactores de las NOS. Alderton utilizó como criterio de selectividad la potencia en condiciones idénticas en un rango fisiológico (Alderton et al. 2001). Según este criterio, los inhibidores son selectivos frente a una isoforma en concreto cuando se haya demostrado su eficacia sobre isoformas aisladas, e idealmente sobre tejidos o in vivo. Su clasificación de selectividad sería la siguiente: Inhibidores no selectivos: cuando su selectividad es menor a una diferencia de 10 veces su potencia, debido a las dificultades inherentes a su uso. Inhibidores parcialmente selectivos: aquellos que tienen una diferencia en su potencia de inhibición entre 10 y 50 veces. Inhibidores altamente selectivos: los que muestran una diferencia en la potencia de inhibición de diferentes isoformas entre 50 o 100 veces.

Una clasificación práctica de los tipos de inhibidores es la efectuada por Salerno (Salerno et al. 2002), que los divide según su estructura química, tal y como se muestra en la siguiente tabla:

(A) Basados en aminoácidos		
	(1) Análogos a la L-arginina	
	(1) Análogos a la L-arginina con restricciones conformacionales	
	(1) Dipéptidos	
(B) No Basados en aminoácidos		
	(1) Compuestos amidínicos	(a) Guanidinas
		(b) Isotioureas
		(c) Amidinas simples
	(1) Compuestos heterocíclicos	(a) Indazol
		(b) Imidazol
		(c) Análogo de la BH4

Tabla 12. Tipos de inhibidores de las NOS según su estructura química (Salerno et al. 2002)

La primera aproximación que se hizo al buscar inhibidores, fue trabajar con inhibidores basados en aminoácidos. Los primeros inhibidores que se diseñaron modificaban ligeramente la estructura del sustrato de las NOS. Eran inhibidores análogos a la arginina, en los que se sustituía el grupo guanidina. Como el sitio activo presenta dominios muy conservados (todos los residuos que están en contacto directo con el sustrato se conservan en todas las isoformas, con la única excepción es un residuo Asp en nNOS e iNOS, que es Asn en la eNOS), encontrar inhibidores específicos de este modo era una tarea difícil (Li & Poulos 2005). Entre estos inhibidores se encuentran la N-metil-L-arginina (L-NMA) y la N-nitro-L-arginina (L-NNA). Son inhibidores potentes (IC50 en el rango nM-uM), más para la eNOS y la nNOS que para la iNOS, pero son poco selectivos. Se consiguió una mejor selectividad para la nNOS frente a la eNOS al alquilar la L-tiocitrulina, un análogo de la L-citrulina. Esta aproximación mostraba el mismo orden de potencia frente a las tres isoformas; entre estos inhibidores, S-metil-L-tiocitrulina (L-SMTC) es más potente contra la nNOS que contra la iNOS y la eNOS. Aunque estos compuestos siguen siendo útiles en el estudio de las funciones de las NOS, y L-NMA y L-NNA son efectivos en pacientes de choque séptico, la poca selectividad entre las isoformas hizo que se buscaran nuevos inhibidores. Apareció entonces la N-propil-L-arginina (L-NPA), un inhibidor competitivo y selectivo de la nNOS frente a la iNOS y la eNOS. Este derivado demostró que era posible inhibir selectivamente una isoforma de la NOS modificando un grupo guanidina de la L-Arg, y que el tamaño y la geometría de la cadena secundaria eran muy importantes para la selectividad (Salerno et al. 2002).

Otra aproximación fueron los ***análogos a la L-Arg con restricciones conformacionales***. En este tipo de aminoácidos se introducen estructuras rígidas que impiden la rotación de la cadena de metileno de la L-Arg. La idea era aumentar la selectividad en el sitio de unión a la arginina y estudiar la mejor orientación de unión, pero este tipo de restricciones no permitía que las moléculas se unieran con la orientación adecuada para dar una buena selectividad, haciendo de ellos unos inhibidores débiles (Salerno et al. 2002).

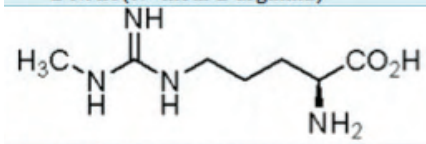
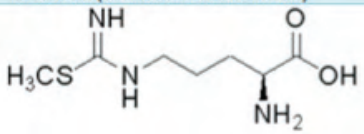
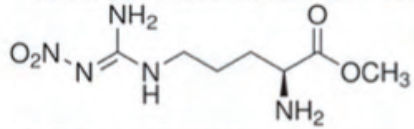
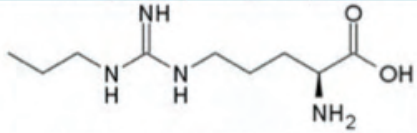
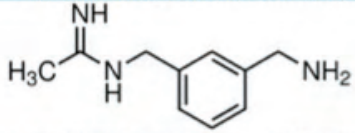
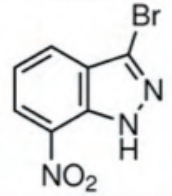
Los inhibidores más selectivos que se fueron diseñando no podían basarse únicamente en la zona de interacción con el grupo guanidino, y por eso se empezaron a utilizar ***dipéptidos*** (Li & Poulos 2005), cuyo diseño partía de inhibidores ya conocidos. Algunos de estos dipéptidos consiguieron una gran potencia inhibitoria y buena selectividad para la nNOS (Salerno et al. 2002).

También se diseñaron ***inhibidores no basados en amino ácidos*** (Li & Poulos 2005), que ampliaban su estructura al canal de acceso al substrato: presentaban una mayor diversidad de aminoácidos, y podían explorar otras zonas para construir la especificidad.

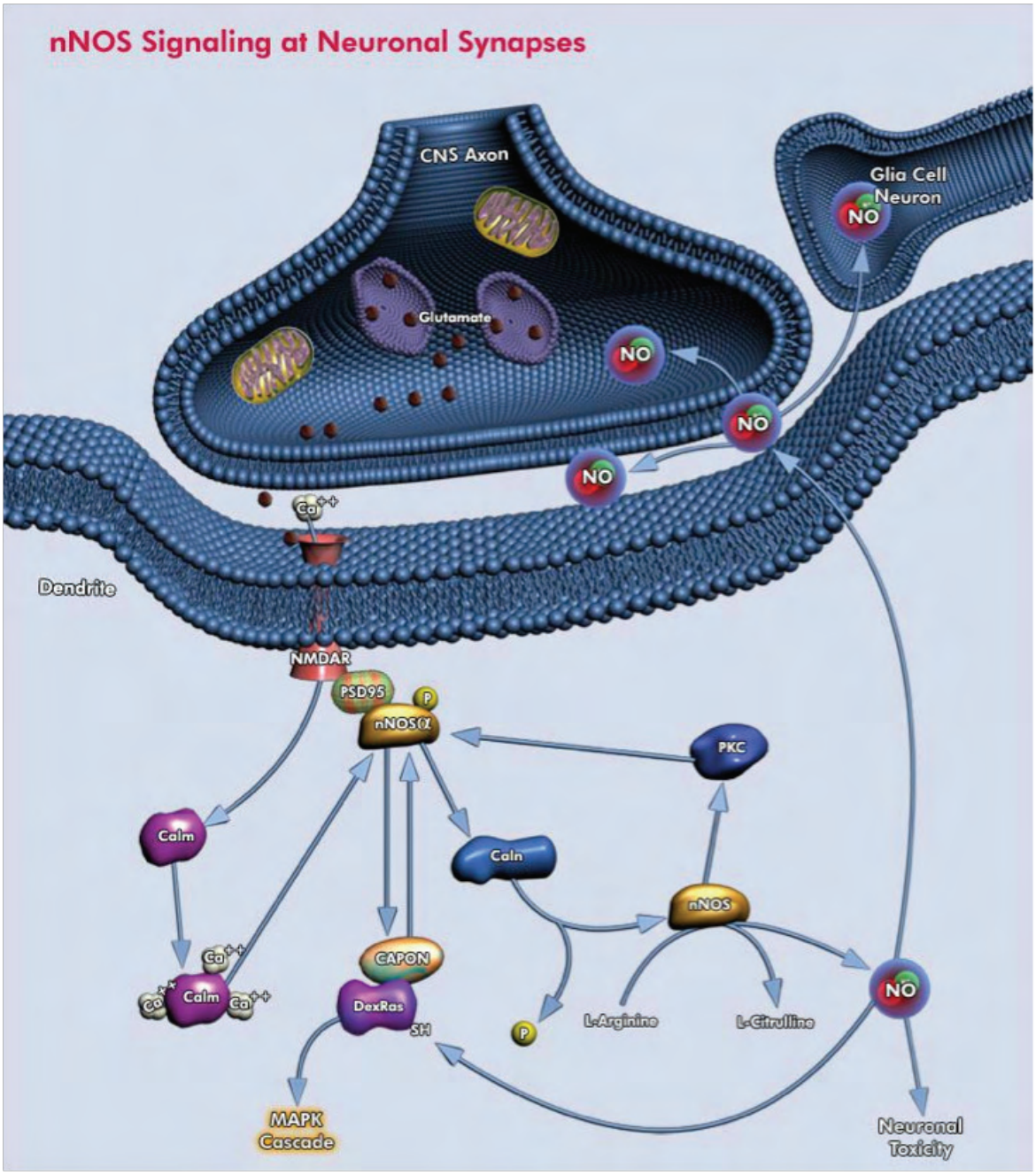
Los ***compuestos amidínicos*** son un tipo de inhibidores no basados en aminoácidos que contienen un carbono carbamida unido a nitrógeno (guanidina), azufre (isotioureas) u otro carbono (amidinas simples). Las ***guanidinas*** son inhibidores débiles, pero el grupo funcional guanidina puede mimetizar la L-Arg en el reconocimiento del sitio de unión. El más conocido es la aminoguanidina. Los inhibidores con ***isotioureas*** son inhibidores potentes y muy selectivos de la iNOS frente a la eNOS. Al comparar la potencia y la selectividad entre los inhibidores basados y no basados en aminoácidos, llegaron a la conclusión de que no era necesario un grupo funcional basado en aminoácidos, y se empezaron a desarrollar derivados de ***amidinas simples***, como una simplificación del grupo funcional guanidino del aminoácido análogo. Entre todas estas amidinas destaca la N-(3-(aminometil)bencil)acetamidina, denominado 1400W, una simplificación de la bisisotiourea, inhibidor muy selectivo de la iNOS frente a la eNOS y la nNOS (Salerno et al. 2002).

Los ***compuestos heterocíclicos*** son un tipo de inhibidores no basados en aminoácidos que contienen en su estructura compuestos heterocíclicos: indazoles, imidazoles y análogos de la BH4. Los ***indazoles*** no presentan selectividad enzimática in vitro, aunque el 3-Br-7-NI, 3-bromo-7-nitroindazol sí presenta selectividad para la nNOS in vivo; actúan reduciendo la afinidad de la nNOS por la BH4 y la L-Arg, desestabilizando el dímero (Zhou & Zhu 2009). Los inhibidores con ***imidazoles*** se empezaron a desarrollar a partir de la evidencia de que las NOS tienen un sitio hemo muy similar al citocromo P450, ya que se sabía que los imidazoles inhibían la actividad de proteínas con grupos hemo. Del mismo modo, se realizó una aproximación con ***análogos a BH4***, con resultados prometedores.

Tabla 13. Inhibidores de las NOS utilizados en esta tesis (Knowles & Moncada 1994; Garvey et al. 1997; Alderton et al. 2001; Salerno et al. 2002; Ishikawa et al. 2003; Kopincová et al. 2012).

Compuesto	Estructura / Tipo de inhibición	IC50(uM)			Ki (nM)			Especificidad		
		nNOS	eNOS	iNOS	nNOS	eNOS	iNOS	nNOS/ eNOS	nNOS/ iNOS	eNOS/ iNOS
L-NMA (N^G-metil-L-arginina)										
	Análogo a la L-arginina	10	5,9	14	180	400	6000	1,7	0.7	0.4
	Se une competitivamente al sitio de unión de la L-arginina. Reversible.	Inhibidor potente, pero poco selectivo. Se da de manera natural en el cuerpo.								
L-SMTC (S-metil-L-tiocitrulina)										
	Análogo a la L-arginina				1,2	11	34	10	28	
	Se une competitivamente al sitio de unión de la L-arginina. Irreversible (o reversibilidad muy lenta)	Parcialmente selectivo para la nNOS sobre la eNOS y la iNOS. Selectividad dependiente del tiempo. En experimentos en enzimas aisladas, pero no tan eficaz in vivo (2). En estudios in vivo de Parkinson L-SMTC muestran protección.								
L-NAME (N^G-nitro-L-arginina metil ester)										
	Análogo a la L-arginina	0,29	0,35	3,1	15	39	4400	1,2	11	
	Se une competitivamente al sitio de unión de la L-arginina. Reversible.	Parcialmente selectivo para eNOS y nNOS. Proviene de la esterificación del N-nitro-L-arginina (L-NNA), y tiene que ser hidrolizado para ser activo. Mayor solubilidad en agua que L-NNA, pero menor potencia. Muy utilizado in vivo e in vitro. A largo plazo, produce un aumento en la expresión y la actividad de NOS. También es un antagonista de los receptores muscarínicos de acetilcolina.								
L-NPA (N$^\omega$-propil-L-arginina)										
	Análogo a la L-arginina	0,057	8,5	180	57	$1,8x10^5$	8500	150	3000	
	Se une competitivamente al sitio de unión de la L-arginina	Inhibidor potente y selectivo para la nNOS, a diferencia de los otros inhibidores basados en la L-arginina.								
1400W (N-(3-(Aminometil)bencil)acetamidina)										
	Amidina simple	7,3	1000	0,23	2000	50000	7	>130		
	Competitiva con L-arginina. Dependiente de NADPH. Irreversible.	Potente y muy selectivo para la iNOS frente a la eNOS y la nNOS. Penetra en células y tejidos. La inhibición de las isoformas constitutivas es reversible, pero no la inducible, donde es muy lento. Es tóxico in vivo a muy altas concentraciones.								
3-Br-7-NI (3-Bromo-7-nitroindazol)										
	Indazol	0.17	0.86	0.29						
	Unión competitiva con los sitios de L-arginiga y BH4.	Proviene del 7-nitroindazol. In vitro es más potente frente a la nNOS, pero in vivo también es potente frente a la iNOS.								

Resumen de la señalización de las óxido nítrico sintasas

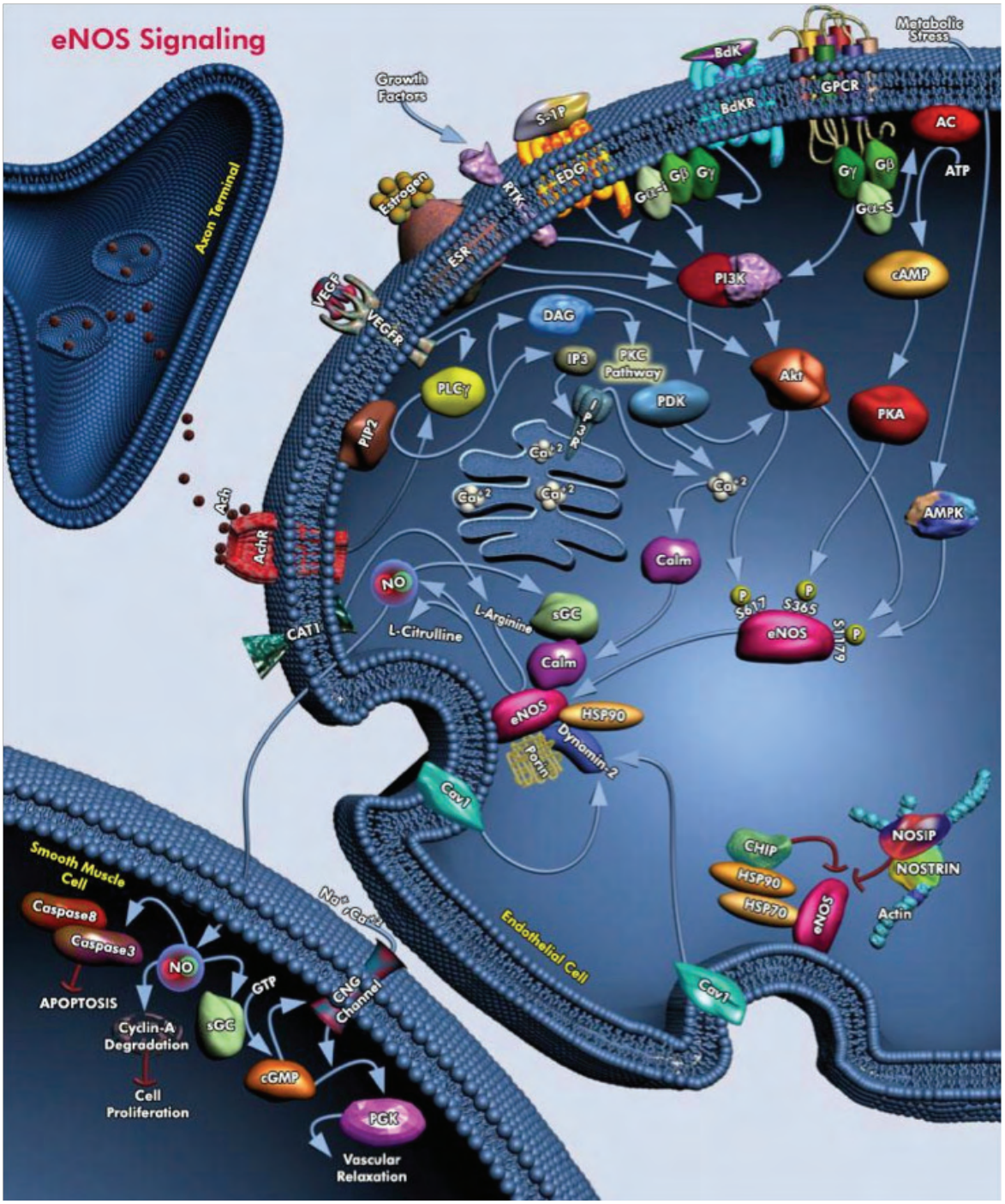

nNOS Signaling at Neuronal Synapses
CNS Axon
Glia Cell
Neuron
NO
Glutamate
NO
NO
NO
Ca++
Dendrite
NMDAR
PSD95
P
nNOSα
PKC
Calm
Caln
nNOS
Ca++
CAPON
Ca++
Calm
Ca++
DexRas
SH
P
L-Arginine
L-Citrulline
NO
MAPK
Cascade
Neuronal
Toxicity

8. Señalización y resumen de las NOS

8.1. Señalización y resumen de nNOS

Señalización y resumen de la nNOS (todos los créditos corresponden a Qiagen (www.qiagen.com)). La isoforma nNOS se encuentra en las membranas post-sinápticas asociada al receptor ionotrópico de glutamato NMDA. El glutamato es el principal neurotransmisor excitatorio en el cerebro, y la forma más efectiva de activar la biosíntesis de NO en el cerebro (Schwarte & Godfrey 2004). El principal regulador de la actividad de la nNOS es el Ca^{2+} citosólico, que activa la nNOS mediante su interacción con CaM (en la imagen aparece como Calm) y calcineurina (en la imagen, Caln). La llegada de un potencial de acción activa los canales de Ca^{2+}, dependientes de voltaje en el neurilema, y estimula la liberación de Ca^{2+} de sus almacenes intracelulares. Esto eleva la concentración de Ca^{2+} citosólico necesarias para la unión de CaM con la nNOS, activando la enzima. Cuando la concentración de Ca^{2+} desciende, se disocia de la CaM, que a su vez se disocia de la nNOS, permitiendo de este modo la activación y desactivación de la enzima (Jobgen et al. 2006). Otro mecanismo de regulación de la actividad de la nNOS es la fosforilación. La actividad catalítica de la enzima disminuye tras ser fosforilada por la PKC dependiente de cAMP, o PKII dependiente de Ca^{2+}-CaM (Tidball & Wehling-Henricks 2004). Este proceso se da en la mayoría de las neuronas nitrérgicas periféricas y en algunas neuronas nitrérgicas centrales. De todos modos, en el SNC la síntesis de NO se regula principalmente por la entrada de Ca^{2+} a través de canales mediados por receptor, principalmente tras la estimulación post-sináptica del NMDAR por glutamato. También se ha observado que la reactividad del NO en cerebro y músculo depende de la asociación de la nNOS con complejos proteicos específicos en las neuronas y los miocitos. Este tipo de interacciones permiten la integración del NO en varias cascadas de transducción en tipos celulares específicos. En el cerebro, el principal producto de splicing es la nNOS-a, una variante de 160kDa que contiene el dominio de unión PDZ en el extremo N-terminal, que se ancla a la densidad post-sináptica próxima al NMDAR. El dominio PDZ de la nNOS se une a un dominio similar de la proteína post-sináptica PSD-95, que a su vez se une a la cola citosólica del NMDAR. Estas interacciones moleculares explican como la entrada de Ca^{2+} a través del NMDAR está eficientemente acoplada a la síntesis de NO y su actividad. Tras su síntesis post-sináptica, el NO difunde al terminal pre-sináptico e interacciona con la GC, aumentando los niveles de cGMP (Y. Xu & Tao 2004). Este complejo de nNOS localizado en la membrana también se une a vías de transducción de señales citoplasmáticas, interaccionando físicamente con DexRas1 y con la proteína adaptadora CAPON, lo que activa la cascada de MAPK y regula la transcripción nuclear. CAPON compite con la nNOS por los dominios PDZ, uniéndose a la enzima y forzando su disociación de la membrana plasmática. De este modo, CAPON determina la cantidad de nNOS anclada a la membrana plasmática, regulando así la formación de NO en las neuronas del SNC. Además, CAPON une la nNOS a otras moléculas, como DexRas1. nNOS también regula la transmisión sináptica y la señalización intercelular, S-nitrosilando el NMDAR, lo que produce una regulación negativa. Además, la vida media de la nNOS-a está regulada por la calpaina, una proteína sensible a la concentración de Ca^{2+} (Ségalat et al. 2005). La nNOS también se puede inhibir por PIN, una proteína muy conservada. Inicialmente se pensó que PIN desestabilizaba los dímeros de nNOS, inhibiendo la síntesis de NO, pero recientemente se ha sugerido que PIN podría funcionar como una proteína transportadora axonal de la nNOS. La nNOS también se puede inhibir por interacción con cav-1 y Cav-3, que desplazan la CaM de la nNOS (Schwarte & Godfrey 2004).

El NO es un mensajero relacionado con la regulación de varios procesos fisiológicos, como la contractilidad muscular, reactividad plaquetaria, neurotransmisión central y periférica y las acciones citotóxicas de las células inmunes. A nivel celular, la señalización por NO es esencial para la plasticidad neuronal: tanto para la PLP en el hipocampo como para la DLP en el cerebelo. El NO producido por las neuronas también juega un papel fundamental en la regulación del flujo sanguíneo. La función del NO en el cerebro no está tan clara, pero probablemente esté relacionado con un aumento local del flujo sanguíneo, ya que esta respuesta se inhibe por los inhibidores de la NOS. Se han observado niveles particularmente altos de nNOS en los nervios vasodilatadores que inervan los grandes vasos cerebrales. El NO producido por las neuronas también media la erección peneana, regulando el flujo sanguíneo. El NO es a la vez protector y perjudicial en la isquemia focal, y aumenta el daño asociado con las primeras fases del infarto: las grandes cantidades de NO que se producen en la isquemia cerebral median el daño neuronal de varias formas de infarto (Danson et al. 2005). También está relacionado con la neurodegeneración en varias enfermedades, como Parkinson, Alzheimer, esclerosis lateral amiotrófica (ELA) y Huntington. La señalización por NO también está implicada en enfermedades musculares como la distrofia muscular de Duchenne. Por todo ello, la regulación farmacológica de la síntesis de NO es una importante estrategia en el tratamiento de enfermedades musculares y neurodegenerativas.

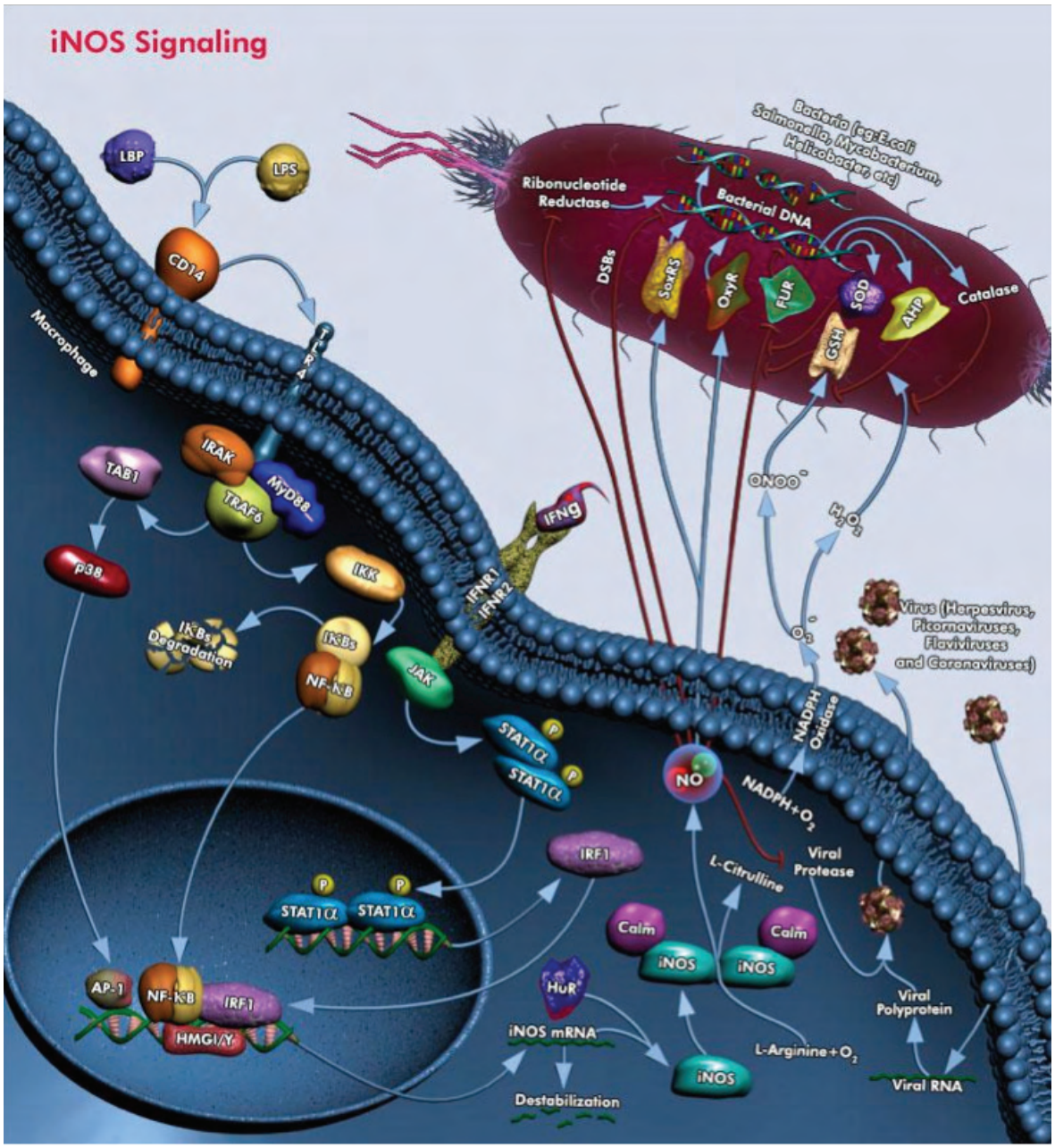

eNOS Signaling
Axon Terminal
Growth Factors
Estrogen
S-1P
EDG
RTK
ESR
BdK
BdKR
GPCR
Metabolic Stress
AC
ATP
cAMP
PI3K
DAG
IP3
PKC Pathway
PDK
Akt
PKA
AMPK
PLCγ
PIP2
VEGF
VEGFR
Ach
AchR
CAT1
NO
L-Citrulline
L-Arginine
sGC
Calm
eNOS
HSP90
Dynamin-2
Porin
Cav1
S617
S365
S1179
CHIP
HSP70
NOSIP
NOSTRIN
Actin
Endothelial Cell
Smooth Muscle Cell
Caspase8
Caspase3
APOPTOSIS
Cyclin-A Degradation
Cell Proliferation
GTP
CNG Channel
cGMP
PGK
Vascular Relaxation

8.2. Señalización y resumen de eNOS

Señalización y resumen de la eNOS (todos los créditos corresponden a Qiagen (www.qiagen.com)).
La producción de NO por las células endoteliales se ve estimulada por fuerzas mecánicas como el estrés hemodinámico y factores humorales como factores de crecimiento, hormonas, incluyendo acetilcolina (ACh), VEGF (Vascular Endothelial Growth Factor), Bdk (Bradiquinina), estrógeno, S-1P (Sphingosine-1Phosphate), H_2O_2 y angiotensina-II. La eNOS es una proteína de membrana doblemente acetilada, anclada a las caveolas del plasmalema endotelial mediante interacción con la proteína estructural de las caveolas, Cav-1. La CaM revierte la inhibición de la eNOS por Cav-1, disociando la eNOS de la caveolina. Este mecanismo regulatorio también se modifica por HSP90, que se une a la eNOS y facilita el desplazamiento de de Cav1 por CaM. Además de estas interacciones proteicas que modulan la unión de CaM, otras cascadas de señalización celular regulan la actividad de la eNOS. Fisiológicamente, las células endoteliales están expuestas a fuerzas hemodinámicas. El estrés hemodinámico activa varias rutas de transducción de señales vía proteínas G (Gs), como PI3K, PDK (Phosphoinositide-Dependent Kinase) y AC (Adenylate Cyclase) vía cAMP (cyclic Adenosine Monophosphate), que activan la eNOS, fosforilándola en residuos serina (S617 y S1179 para Akt, y S635 y S1179 para PKA). Otros estímulos adicionales como VEGF, estrógenos, S-1P y Bdk, unen sus receptores cognados RTKs, VEGFR, ESR, EDG and BdkR) y estimulan PI3K/Akt. Sin embargo, también activan la PLC-g (Phospholipase-C) y PIP2 (Phosphatidyl Inositol 4, 5-Bisphosphate) para aumentar sus niveles de Ca^{2+} citoplasmático. El aumento de los niveles de Ca^{2+} citoplasmático activan la CaM, que se une al dominio CaM en la eNOS para promover la alineación de los dominios oxigenasa y reductasa de la eNOS, permitiendo la síntesis de NO. Además, CaM activa CaMKII, que fosforila la eNOS en S1179. Finalmente, el estrés metabólico promueve la ruptura de ATP, lo que estimula la AMPO (AMP Kinase) para fosforilar la eNOS en el residuo S1179, por PKA. Esta fosforilación también está asociada a un aumento de la actividad de la enzima. Otras proteínas cuyos residuos están asociados con un aumento de la actividad de la eNOS o la liberación de NO son la Dinamina-2 y la porina. El transportador de arginina CAT-1 está directamente asociado con la eNOS en las caveolas, lo que asegura un correcto suministro de sustrato.

La eNOS puede interaccionar con varias proteínas en estados más y menos activos. La miristoilación de la eNOS es un proceso co-traduccional y ancla la eNOS a las membranas celulares, donde se palmitoila. Esta lipidación promueve la asociación de la eNOS con las membranas celulares y es esencial para unirla a las rutas de transducción de señales en las que participa. La eNOS N-miristoilada y palmitoilada, unida a la membrana, se asocia con Cav-1 y HSP90. CHIP (C-terminal HSP70-Interacting Protein) interacciona tanto con HSP70 como con HSP90, regulando negativamente el tráfico de la eNOS al complejo de Golgi. Por otro lado, NOSIP, y NOSTRIN (Nitric Oxide Synthase Traffic Inducer) regulan negativamente la localización de eNOS en la membrana plasmática. La activación aguda de la eNOS en los vasos sanguíneos en respuesta a agonistas como la ACh o Bdk activa la sGC en las células del músculo liso, la producción de cGMP y la degradación de la ciclina-A. El aumento de los niveles intracelulares de cGMP afecta al tono vascular, disminuyendo la concentración intracelular de Ca^{2+} libre, activan la PKG y fosforilando la HSP20, que regulan la fuerza uniéndose a los filamentos finos e inhibiendo el movimiento de los puentes cruzados (Minshall et al. 2003). La nitrosilación de la caspasa3 y la caspasa8 inactiva las proteínas, inhibiendo la apoptosis.

La eNOS es un importante regulador de la homeostasis cardiovascular ya que es la principal fuente de NO en las células endoteliales. La eNOS juega un papel crucial en la vasodilatación de los vasos sanguíneos, y por lo tanto en la regulación de la presión arterial. Además, el NO liberado por el endotelio modula otros procesos como la agregación plaquetaria, la adhesión de plaquetas y leucocitos al endotelio, la generación de endotelina-1, la proliferación de células del músculo liso vascular y la angiogénesis. Dada la importancia del NO en estos procesos, se considera que las anormalidades en la producción de NO vascular contribuyen a la patogénesis de ciertas enfermedades vasculares como la aterosclerosis y la hipertensión (Ho et al. 2006; Boo & Jo 2003).


iNOS Signaling
Bacteria (e.g. E.coli Salmonella, Mycobacterium, Helicobacter, etc)
Ribonucleotide Reductase
Bacterial DNA
DSBs
SoxRS
OxyR
FUR
SOD
AHP
Catalase
GSH
LBP
LPS
CD14
TLR4
Macrophage
IRAK
TAB1
MyD88
TRAF6
p38
IKK
IκBs Degradation
IκBs
NF-κB
IFNg
IFNR1
IFNR2
JAK
STAT1α
STAT1α
ONOO⁻
H2O2
O2⁻
Virus (Herpesvirus, Picornaviruses, Flaviviruses and Coronaviruses)
NADPH Oxidase
NO
NADPH+O2
Viral Protease
L-Citrulline
IRF1
Calm
iNOS
iNOS
Calm
HuR
iNOS mRNA
Destabilization
iNOS
L-Arginine+O2
Viral Polyprotein
Viral RNA
AP-1
NF-κB
IRF1
HMGI/Y

8.3. Señalización y resumen de iNOS

Señalización y resumen de la iNOS (todos los créditos corresponden a Qiagen (www.qiagen.com)).

La iNOS se identificó y caracterizó por primera vez en macrófagos, como una isoforma de la NOS inducible por endotoxinas o citoquinas, que genera grandes cantidades de NO, probablemente para matar o inhibir el crecimiento de microorganismos patógenos o tejido neoplásico (Kadowaki et al. 2004; Petruson et al. 2005). Se ha demostrado la presencia de la iNOS en virtualmente todos los tipos celulares en los que se ha buscado, incluyendo células endoteliales, fibroblastos, células del músculo liso vascular y cardiomiocitos. La actividad catalítica de la iNOS está regulada por la disponibilidad de sustrato, CaM, L-Arg, y los cofactores NADPH y BH4. El NO y el O2–, ambos producidos por la iNOS, son efectores radicales del sistema inmune innato que pueden inhibir directamente la replicación de patógenos.

Aunque la actividad de la iNOS no depende de la concentración de Ca2+ intracelular, varios estímulos extracelulares pueden activar diferente vías de señalización que convergen para iniciar la expresión de la iNOS. Los componentes de la pared de bacterias y hongos inician la cascada de señalización inmunitaria innata para expresar iNOS. El LPS es un componente de la parece de bacterias gran-negativas, que se une a LBP (LPS-Binding Protein), que lleva el LPS a un receptor de gran afinidad, el CD14. TLR4 (Toll-Like Receptor-4), junto con la proteína extracelular MD2 interacciona con el complejo CD14-LPS y activa la cascada de señalización intracelular mediante unos adaptadores, IRAK (Interleukin-1 Receptor-Associated Kinase) y MyD88 (Myeloid Differentiation Primary Response Gene-88), que a su vez activan TRAF6 (TNF Receptor-Associated Factor-6), TAB1 (TAK1-Binding Protein-1) and p38. La activación de TLR4 por LPS lleva a la fosforilación de IKK, que fosforila a su vez I-kB y libera el factor de transcripción NF-kB. NF-kB transloca desde el citoplasma hasta el núcleo, donde interacciona con los elementos kB de la región flanqueante 5 de la iNOS, iniciando su transcripción. (Kadowaki et al. 2004; Davis et al. 2005; Mizel et al. 2003). Las citoquinas liberadas por células infectadas también activan la producción de NO, incluyendo TNF-a e IL-1β. IFN-γ interacciona con el complejo IFNR1 (Interferon Receptor-1) IFNR-2, activando las quinasas de las familias JAK y STAT, lo que promueve la síntesis de IRF1 (Interferon Response Factor-1) y la estimulación de la transcripción del mARN de la iNOS. IFN-γ también aumenta de manera sinérgica la inducción de la transcripción de iNOS provocada por LPS, ya que IRF1 interacciona con NF-kB, alterando la conformación del promotor de iNOS. Ciertas proteínas nucleares interaccionan con la familia NF-kB, como las proteínas cromosómicas no histonasHMGI/Y. Aumentan la unión de factores de transcripción como NF-kB y AP-1 a sus sitios de unión mediante interacciones tipo proteína-proteína y ADN-proteína. (Davis et al. 2005; Jang et al. 2004). Otros factores de transcripción como STAT-1ª y HIF1 también regulan la expresión de iNOS.

El NO es un efector anti-bacteriano y puede inhibir la síntesis de ADN bacteriano, rompiendo la doble hélice de ADN. También aumenta la susceptibilidad de las bacterias al daño oxidativo al ADN al bloquear la respiración. El NO reacciona con O2– para dar ONOO– y oxidar lípidos bacterianos para producir nitrotirosina. Algunas bacterias contienen bajas concentraciones de GSH, lo que las hace susceptibles al NO. El NO también tiene un efecto anti-viral en el sistema inmune innato. Puede inhibir la replicación de varios tipos de virus reaccionando con proteasas virales. Aunque la inducción de la iNOS puede proteger el cerebro de ciertas enfermedades infecciosas, los excesivos niveles de NO también pueden ser tóxicos para las neuronas. Se ha propuesto que el aumento en la producción de NO por la inducción de la iNOS es el principal mecanismo por el que las citoquinas median la disfunción contráctil cardiaca y se desarrolla la enfermedad cardiovascular. La sobreexpresión de la iNOS, algo muy corriente en la inflamación crónica, produce grandes cantidades de NO, y sus intermediarios reactivos son mutagénicos, provocando daños en el ADN o disfunción en sus mecanismos de reparación. El NO también está implicado en los procesos de regulación de la tumorigénesis. También se ha observado un aumento en la expresión de la iNOS en tumores de colon, pulmón, faringe, órganos reproductivos, pecho y SNC, además de su implicación en enfermedades inflamatorias crónicas. El desarrollo de inhibidores selectivos de la iNOS podría ser una estrategia para la quimio prevención del cáncer (Gavrilescu et al. 2004; Lala & Chakraborty 2001; Bafica et al. 2005)

C. Células cromafines bovinas

1. Las glándulas suprarrenales

Las glándulas suprarrenales son dos pequeñas formaciones retroperitoneales, situadas en los polos superiores de los riñones, embebidas en tejido adiposo. Están irrigadas por las arterias suprarrenales superiores, media e inferior. Son relativamente planas, de menos de un centímetro de grosor, pero no son simétricas: la glándula derecha tiene forma triangular, y la izquierda de semiluna. Miden aproximadamente dos centímetros en la base y cinco centímetros en el vértice. En conjunto, en los adultos humanos pesan entre 15 y 20 gramos. Las principales funciones de las glándulas suprarrenales son mantener constante el medio interno del organismo y realizar los cambios apropiados en su fisiología en respuesta al estrés agudo, la lesión o la carencia prolongada de agua y alimentos.

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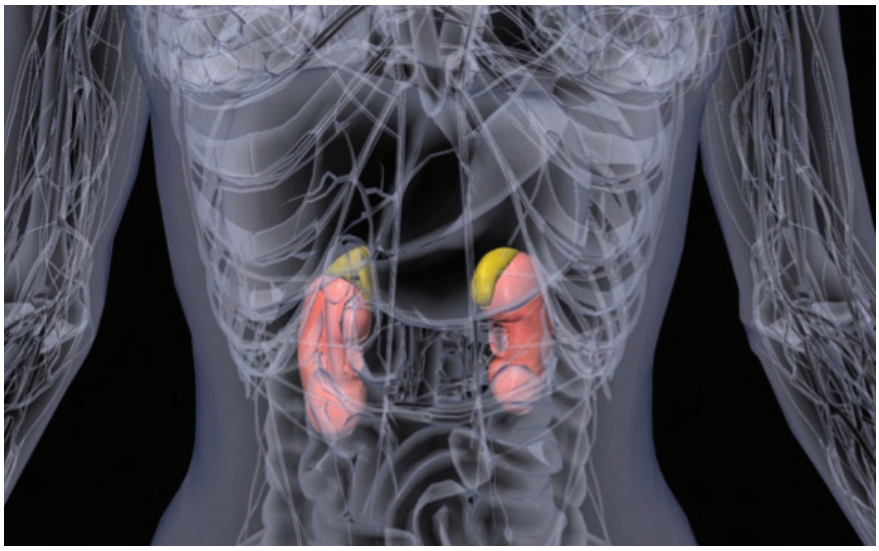

Imagen 30. Localización anatómica de las glándulas suprarrenales y los riñones. Imagen realizada con el programa Biodigital Human, de BIODIGITAL SYSTEMS LLC, https://www.biodigitalhuman.com.

En un corte transversal se diferencian claramente dos partes: la parte más externa, una corteza amarilla y gruesa, que supone entre el 80 y el 90% del total de la glándula, y la parte interna, compuesta por una médula gris y delgada, que representa el 10-20% restante. A pesar de estar organizadas como una glándula única, corteza y médula son dos ganglios funcionalmente diferentes, y con distinto origen embriológico.

Imagen 31. Corte transversal de glándula adrenal. Izquierda: dibujo esquematizado, donde se distinguen, de fuera hacia dentro, la cápsula, corteza y médula. Derecha: imagen real.

La corteza suprarrenal se desarrolla en el mesotelio abdominal, y rodea la médula durante la embriogénesis. Estructuralmente está separada de los órganos próximos por una cápsula de tejido conjuntivo, y en ella se distinguen tres zonas concéntricas: una capa externa y delgada, inmediatamente por debajo de la cápsula, denominada zona glomerular, que constituye el 15% del volumen total de la corteza, donde se producen mineralcorticoides; una zona fasciculada intermedia y ancha, que ocupa el 78% del volumen de la corteza, encargada de la síntesis de glucocorticoides; y una zona reticular interna, adyacente a la médula, que conforma el 7% restante, en la que se sintetizan hormonas sexuales.

La médula comprende el 20% de la glándula. Se origina en la cresta neural y, en humanos, no se diferencia de la corteza hasta que ésta se atrofia, durante las primeras semanas postnatales. Secreta catecolaminas, que ejercen funciones similares a las de la estimulación simpática directa (Fawcett 1995).

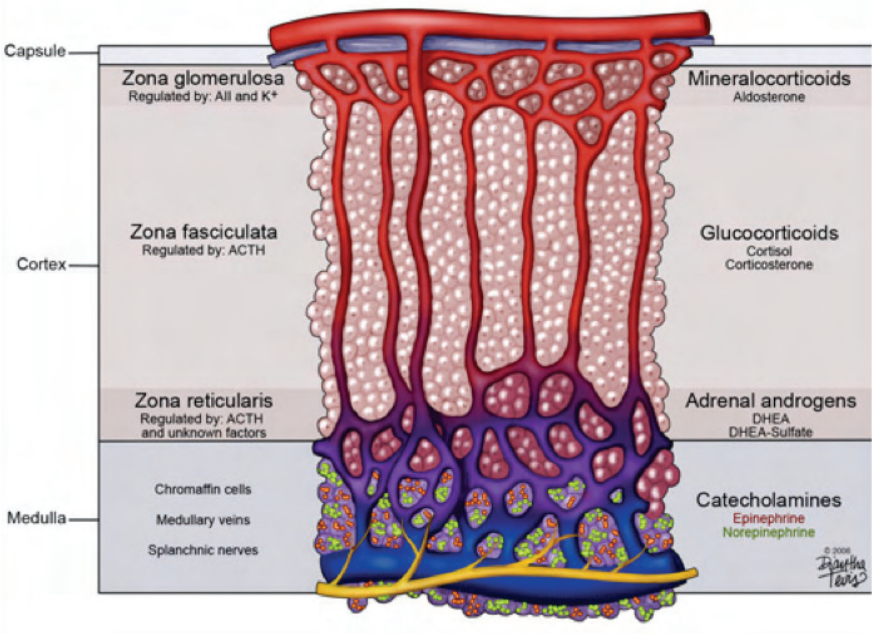

Imagen 32. Corte transversal de la glándula adrenal. En el panel de la izquierda se esquematizan las principales diferencias anatómicas y bioquímicas. El panel de la derecha muestra el tipo de hormonas, y las hormonas secretadas. Imagen tomada de la Georgia Regents University.

2. La médula suprarrenal

La médula adrenal es un ganglio simpático modificado, constituido por células postganglionares, que funcionan como neurosecretores clásicos liberando catecolaminas y otras sustancias (NO, pej.) al sistema circulatorio. Tiene una doble estimulación: por los nervios simpáticos y por las hormonas de la médula suprarrenal.

Se considera que la médula adrenal es un ganglio simpático modificado, constituido por células postganglionares que carecen de dendritas y axones. Como tal, sus células funcionan como neurosecretores clásicos, liberando catecolaminas al sistema circulatorio, en respuesta a estímulos de los nervios simpáticos colinérgicos de la médula. Estas estructuras tienen una doble estimulación: de manera directa por los nervios simpáticos, y de manera indirecta por las hormonas de la médula suprarrenal (Mravec 2005).

La médula adrenal libera una mezcla de catecolaminas, 80% epinefrina y 20% norepinefrina, y pequeñas cantidades de otras sustancias como dopamina, ATP, neuropéptidos y NO. Las catecolaminas se liberan de la médula al mismo tiempo que se produce la excitación simpática. Se cree que las células que secretan noradrenalina y adrenalina reciben una inervación diferente y secretan sus respectivas hormonas de forma independiente. Por ejemplo, los estímulos emocionales son especialmente eficaces en la inducción de la liberación de norepinefrina mientras que otros estímulos, como el dolor o la hipoglucemia, favorecen la liberación de epinefrina (Fawcett 1995). La médula suprarrenal produce el 100% de la epinefrina y el 30% de la norepinefrina circulantes. Las catecolaminas liberadas por la médula ejercen las mismas acciones que la estimulación nerviosa directa, con dos particularidades: sus efectos duran de cinco a diez veces más, ya que su retirada de la circulación periférica es muy lenta, tardando entre dos y cuatro minutos en desaparecer; y pueden estimular estructuras del cuerpo no inervadas directamente por fibras simpáticas (Eaton & Duplan 2004). Las células de la médula adrenal se conocen bajo el nombre de células cromafines.

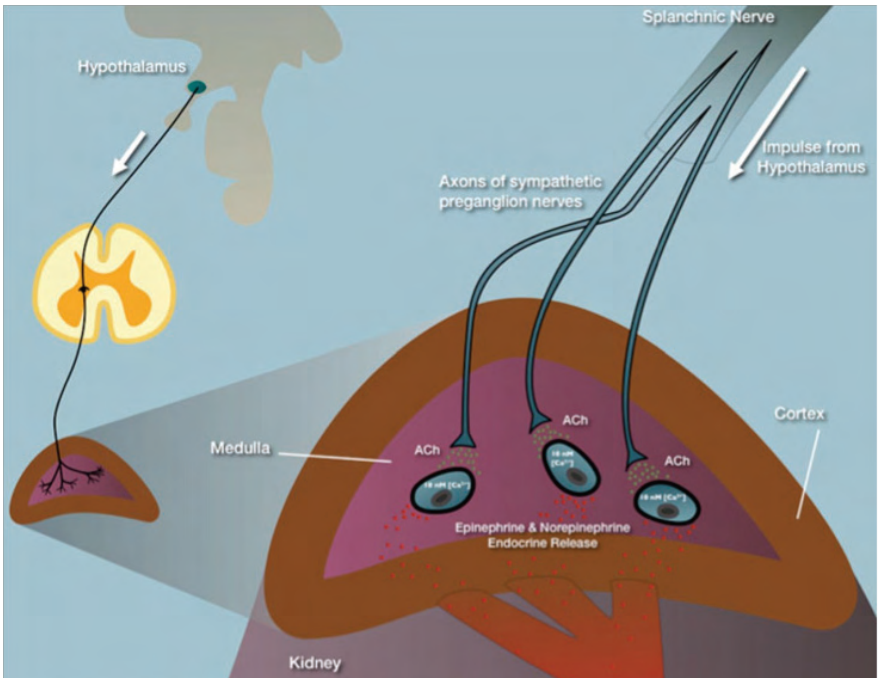

Imagen 33. Respuestas fisiológicas de células cromafines adrenales bovinas ante la inervación nerviosa simpática de la médula adrenal, (Weiss 2012). Entre el nervio esplácnico y las células cromafines se produce una sinapsis colinérgica. Los impulsos nerviosos del hipotálamo se transmiten por los terminales preganglionares simpáticos del nervio esplácnico, inervando la médula adrenal. Los potenciales de acción provocan la liberación de acetilcolina (ACh). Si la liberación de ACh es prolongada, se produce una despolarización de las células cromafines adrenales postganglionares. Esto provoca una activación de las caveolinas y las vías de señalización de calcio (ver más adelante), produciéndose la liberación de catecolaminas.

3. Las células cromafines

3.1. ¿Qué son las células cromafines? Tipos y localización.

Las células cromafines son células neuroendocrinas, consideradas como neuronas simpáticas postganglionares modificadas. Derivan del neuroectodermo y sintetizan, almacenan y secretan catecolaminas (epinefrina, norepinefrina y dopamina) a partir de tiroxina. Estas sustancias se almacenan en los gránulos cromafines, que liberan su contenido por exocitosis, en un proceso dependiente de ATP y calcio.

Las células cromafines son células neuroendocrinas, consideradas como neuronas simpáticas postganglionares modificadas.

A mediados del siglo XIX se observó que había una serie de reacciones químicas que permitían diferenciar la corteza de la médula suprarrenal. Ciertas células se teñían de un color amarillo parduzco cuando se las sumergía en una solución acuosa de sales de cromo. A principios del siglo XX, Alfred Kohn describió como "reacción cromafín" a este cambio de color en presencia de sales de cromo, y especialmente dicromato potásico (Coupland 1965a; Coupland 1965b), y denominó células cromafines a las células en las que se producía este cambio de color. Posteriormente se observó que la reacción cromafín resultaba de la oxidación de las catecolaminas almacenadas, más que de la afinidad al cromo; (en el caso de las células enterocromafines, esta reacción positiva se debe a la serotonina almacenada). Las células que presentan una reacción cromafín positiva se pueden dividir en tres grandes grupos:

- Las células cromafines verdaderas, localizadas en la médula de la glándula suprarrenal;

- Las células enterocromafines del tejido epitelial que tapiza los tractos gastrointestinales y respiratorios. No se consideran células cromafines reales; No derivan de la cresta neural y no pueden convertirse en neuronas simpáticas. Almacenan serotonina.

- Los mastocitos, que almacenan aminas, y se encuentran en los tejidos conjuntivos del intestino, páncreas e hígado.

Las células cromafines verdaderas, o feocromocitos, son elementos derivados del neuroectodermo capaces de sintetizar, almacenar y secretar catecolaminas (adrenalina, noradrenalina y dopamina).

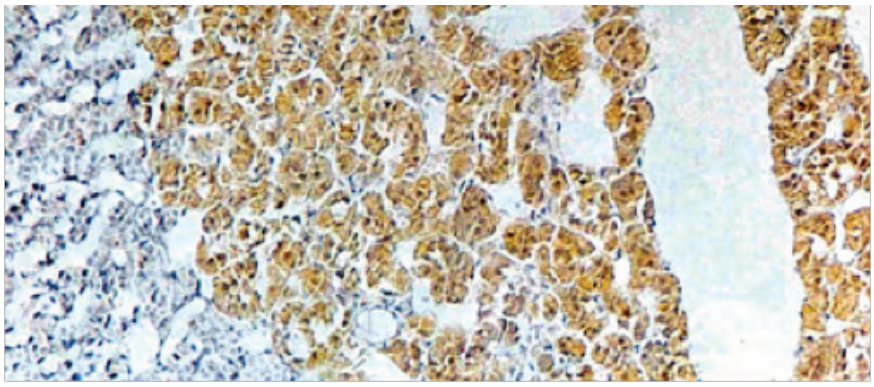

Imagen 34. Células cromafines bajo tinción con sales de cromo

El sistema cromafín comprende grupos de dichas células secretoras de hormonas, asociadas estructural y funcionalmente al sistema nervioso simpático. Este sistema cromafín comprende: a) la médula de las glándulas suprarrenales; b) los cuerpos paraórticos (paraganglios abdominales); c) los paraganglios propiamente dichos; d) ciertas células de los cuerpos carotideos; y e) pequeñas masas de células diseminadas irregularmente y en cantidades variables entre los ganglios de las cadenas simpáticas paravertebrales, los nervios esplácnicos y los plexos autónomos mayores (prevertebrales). El sistema cromafín, por tanto, está íntimamente relacionado con diversos órganos (corazón, hígado, riñones, uréteres, próstata, epidídimo, ovario, etc.) (Unsicker et al. 1997; DeLellis & A. Tischler 1998; S. M. Murphy et al. 2003).

3.2. Síntesis de catecolaminas

Las catecolaminas epinefrina, norepinefrina y dopamina se sintetizan en el interior de las células a partir de tiroxina, en función del estado adrenérgico del sistema, en un proceso regulado por varias enzimas.

En un primer estadio, tiroxina se convierte en Dopa, en un proceso catalizado por la tiroxina hidroxilasa (TH), presente en el citosol de las células cromafines. Esta enzima constituye el paso limitante en la producción de catecolaminas, y está regulada por la PKA, CaMKII y PKC, por procesos de fosforilación, por concentración de calcio o, trans-sinápticamente, por la ACh a través de receptores, entre ellos los de tipo nicotínico.

En un segundo estadio, dopa es rápidamente transformada en dopamina en un proceso mediado por dopamina-decarboxilasa, también en el citosol. Tras esto, la dopamina es transportada a las vesículas donde se encuentra la enzima dopamina-B-hidroxilasa (DBH), encargada de la oxidación de dopamina a noradrenalina; Esta enzima está regulada por transcripción, puesto que su promotor contiene regiones involucradas en la respuesta a TPA, cAMP y c-jun.

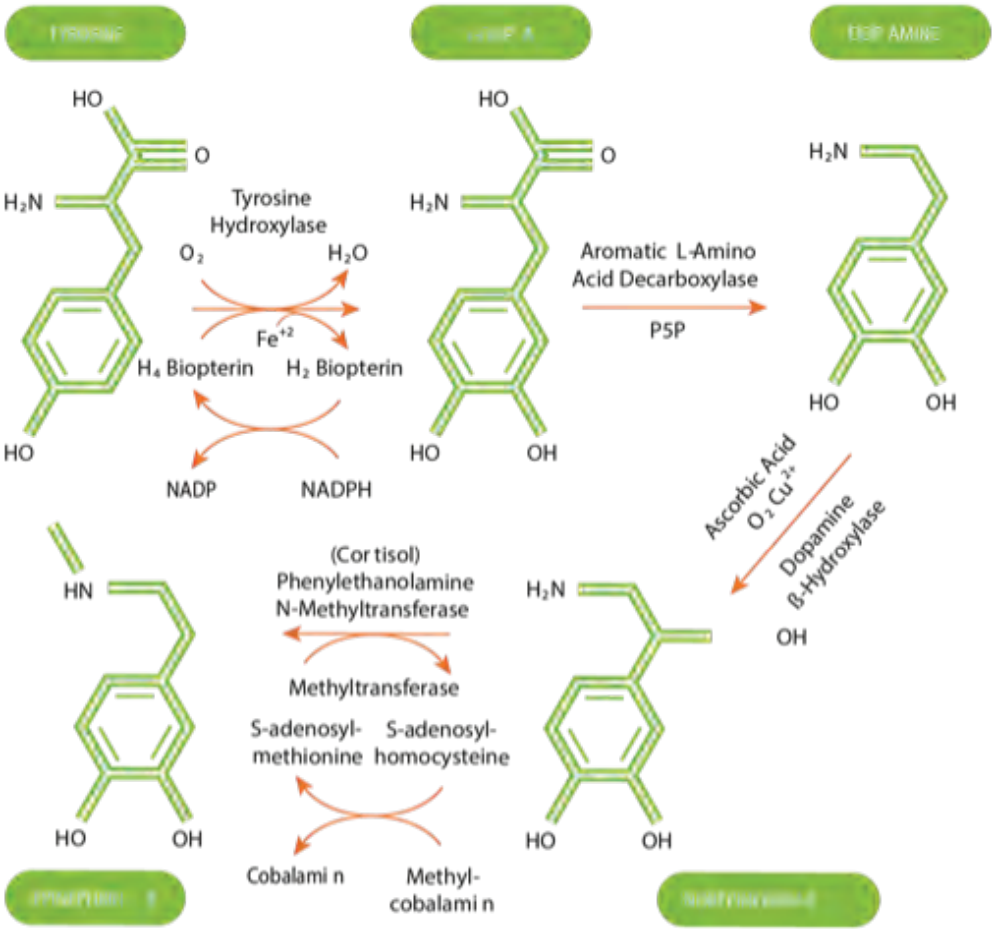

Imagen 35. Síntesis de catecolaminas

En algunas células de la médula adrenal el proceso de síntesis acaba en la producción de norepinefrina. En el 90% de los casos, este proceso consta de una cuarta etapa en la que la norepinefrina se transforma en epinefrina gracias a la feniletanolamina-N metiltransferasa (PNMT). La PNMT se encuentra sólo en el citosol, por

lo que las catecolaminas se desplazan de nuevo desde las vesículas hasta el citosol. La transcripción de PNMT parece estar regulada por neuropéptidos, y mecanismos de señalización (Trifaro 2002; Jacintho & Kovacic 2003).

Los principales marcadores enzimáticos del fenotipo cromafín son la TH y PNMT (Eaton & Duplan 2004).

3.3. Los gránulos cromafines

En 1953 H. Blaschko, A. Welch y N.A. Hillarp descubrieron que la epinefrina y la norepinefrina no estaban libres en el citosol de las células cromafines, sino que se encontraba dentro de organelas, a los que se denominó gránulos cromafines. J. Lever observó que estos orgánulos eran pequeñas vesículas de unos tres micrómetros de diámetro. J. Phillips calculó que cada célula cromafín contiene unos 30.000 gránulos cromafines (Winkler & Westhead 1980).En los gránulos cromafines se almacenan catecolaminas, neuropéptidos, ATP y proteínas cromograninas a concentraciones muy altas: 0,5-1M (Kopell & Westhead 1982; Sen & Sharp 1982; Finnegan et al. 1996; Albillos et al. 1997).

Una célula cromafín tiene un promedio de diez mil gránulos, cantidad suficiente para liberar a la sangre las catecolaminas necesarias para responder a una reacción de "lucha o huída". Unas cuantas células cromafines serían suficientes para aportar todos los estímulos normales y excepcionales a los que se somete el cuerpo. El desgranamiento del 5% de las células cromafines, crea una concentración de catecolaminas en suero de 10-6 molL-1, provocando efectos irreversibles en humanos, como edema pulmonar, paro cardiaco, hipertensión... (Crivellato et al. 2006). Con estos datos es evidente que la liberación de catecolaminas tiene que estar muy regulada.

3.4. Secreción de los gránulos cromafines

Se ha asumido que, con los estímulos adecuados, los gránulos cromafines se dirigen a la superficie celular vía microtúbulos y liberan su contenido por exocitosis (Crivellato et al. 2006). La exocitosis consiste en la fusión de una vesícula con la membrana plasmática, de modo que las sustancias almacenadas en el interior de la vesícula se liberan de modo unidireccional al medio extracelular. Durante los años 60 y 70 se estableció que los únicos requerimientos necesarios para que tenga lugar la exocitosis son la presencia de ATP y calcio (Camacho & Borges 2003), siendo la elevación de calcio intracelular el evento desencadenante (Cheek & Barry 1993).

Se conocen tres tipos de exocitosis: exocitosis típica o de fusión total, exocitosis parcial tipo "kiss-and-run" y exocitosis parcial similar a la que se produce en los mastocitos tipo degranulación escalo-

nada. La existencia de estos tres tipos de exocitosis proporciona a la célula precisión para regular la cantidad de catecolaminas liberadas al medio.

La exocitosis tipo "Full fusión" o "fusión completa" es una manera rápida de liberar el contenido de los gránulos cromafines. Es un evento todo o nada, y probablemente funcione cuando el organismo esté sometido a situaciones de estrés rápidas e imprevistas.
La exocitosis de tipo "kiss-and-run", aunque de controvertido significado fisiológico, está ganando fuerza como mecanismo de secreción de los gránulos cromafines (Crivellato et al. 2006). La membrana del granulo cromafín no se fusiona completamente con la membrana celular, sino que forma un poro de fusión transitorio, que se vuelve a cerrar rápidamente, separando el gránulo de la membrana. De este modo, sólo se expulsa una parte del material almacenado en los gránulos.

El calcio participa en la dinámica de la fusión del poro y en la regulación del fenómeno "kiss-and-run". Se ha demostrado que la variación de los niveles de calcio extracelular puede cambiar el tipo de secreción en las células cromafines entre "full fusión" y "kiss-and-run". El NO está implicado en la regulación de los canales de calcio y en el control del potencial de membrana a través de los canales de K+ activados por Ca2+ (Crivellato et al. 2006; Machado et al. 2000; Machado et al. 2001).

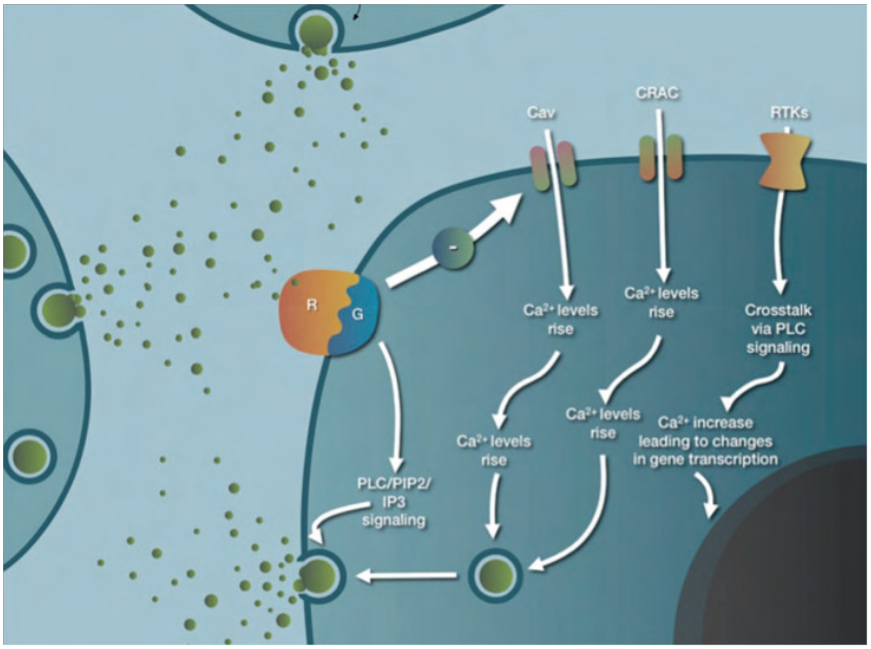

Imagen 34. Transducción de la señalización por calcio en células cromafines bovinas (Weiss 2012). El calcio controla tanto la señalización paracrina como la autocrina. La señalización entre células cromafines bovinas en cultivo se produce por catecolaminas y ATP. Actúan sobre receptores acoplados a proteínas G (RG), que a su vez actúan sobre canales de calcio activados por voltaje (En esta imagen represntado por cav), canales activados por la liberación de calcio (en la imagen, CRAC) y receptores de tirosina quinasas (RTKs). Otras proteínas que se unen o funcionan como sensores de calcio, como la CaM NCS-1, calneurina, CaBP-1 también median estas vías de señalización.

4. Factores de crecimiento

Como se ha mencionado anteriormente, las células cromafines no contienen sólo catecolaminas, sino también otras muchas sustancias como citoquinas y factores de crecimiento. Los factores de crecimiento se descubrieron al observar que eran capaces de prevenir la muerte celular. En ellas se ha estudiado el Factor de crecimiento de fibroblastos (FGF-2), que junto a IGFγ (Insulin-like Growth Factor) e insulina, controla el ciclo celular. El TGF-**B**, que también regula el ciclo celular y la formación de la matriz extracelular. Interleuquinas, neurotrofinas (Factor de crecimiento nervioso (NGF), neurotrofinas 3 y 4/5); Insulina e IGF, y finalmente, cromograninas A y B y secretograninas (Mattson 2003).

5. Receptores en las células cromafines bovinas

Las células cromafines representan un excelente modelo para el estudio de la señalización celular, pues en ellas se han encontrado numerosísimos receptores implicados en la regulación de la exocitosis y localizados en la membrana plasmática (Bunn et al. 2012):

Receptores colinérgicos: nicotínicos, que permiten la entrada de calcio a la célula, aumentando la secreción, y muscarínicos, que regulan el contenido en cGMP. *Receptores glutamatérgicos:* de tipo ionotrópico, que a su vez se clasifican en tipo NMDA y no NMDA (AMPA y KA), y de tipo metabotrópico, que se subclasifican como t-ACPD y L-AP. Receptores adrenérgicos; *Receptores dopaminérgicos*; *Receptores purinérgicos:* tipo P1 o sensibles a adenosina y AMP o tipo P2 o sensibles a ATP y ADP; *Receptores de neuropéptidos; Receptores GABAérgicos; Receptores de citoquinas.*

6. Señalización por citoquinas en las células cromafines bovinas

En la membrana celular de las células cromafines se han encontrado varios receptores específicos de citoquinas (TNF-R2, IL-1R, IL-6R, IFNαR). Las citoquinas parecen estimular la actividad de las MAP quinasas. Las MAPK activas interaccionan con dianas citosólicas y factores de transcripción nucleares. Además, la vía de señalización de cada citoquina contiene reguladores directos de la transcripción: NF-κB (p65/p50) para TNF-α, STAT1/STAT2 y STAT3 para IFN-α e IL-6, respectivamente, con diferentes patrones temporales.

El funcionamiento de las citoquinas se produce siguiendo tres normas: Cada citoquina aumenta la espresion de un grupo concreto de genes, cuyos productos actúan como reguladores directos de esta vía; Cada citoquina regula la actividad de las otras citoquinas, para facilitar la propagación controlada del avance de la inflamación. Cada citoquina aumenta la expresión de varios neuropéptidos. (Ver imagen para más información)

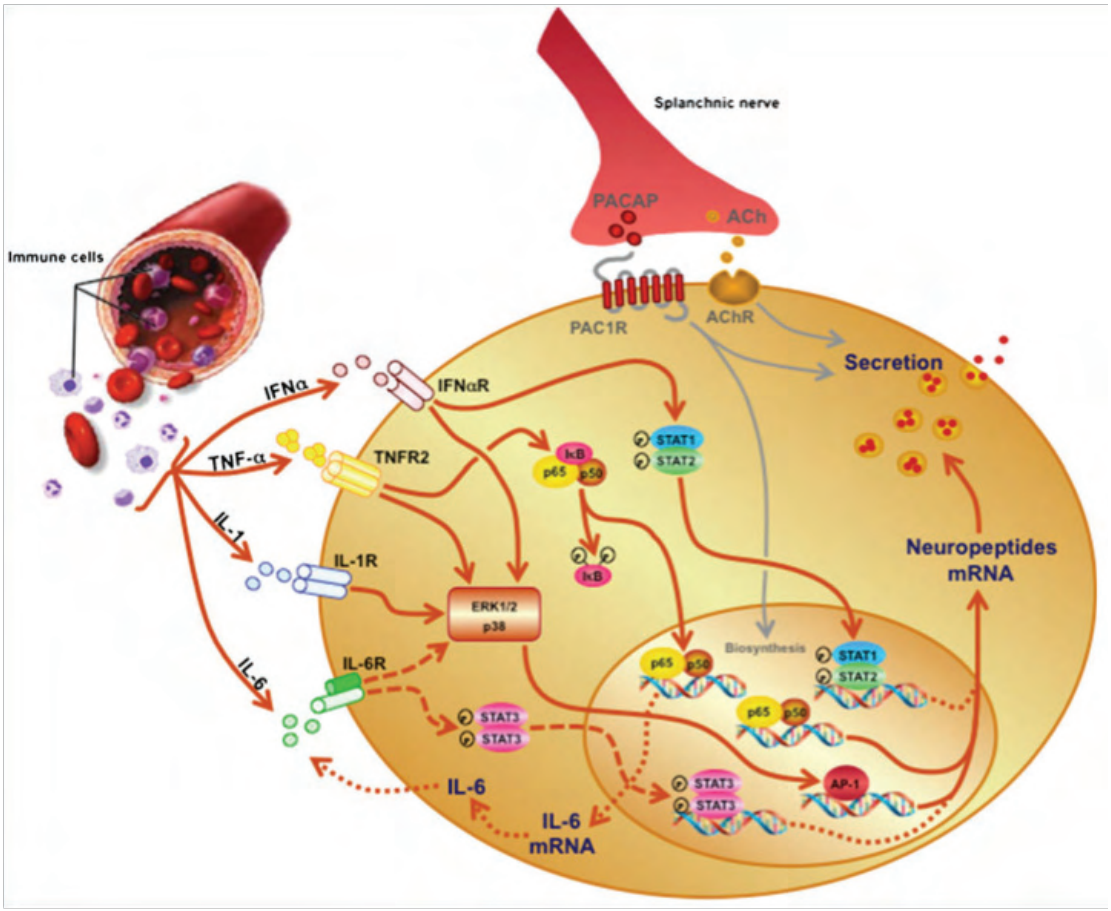

Imagen 35. Rutas de señalización por citoquinas en las células cromafines de la médula adrenal (Bunn et al. 2012)

7. El NO en las células cromafines

Las células cromafines presentan NOS capaces de sintetizar NO.

La presencia de NO en las glándulas adrenales se debe a varios factores: por una parte, los nervios aferentes que llegan hasta las glándulas son capaces de secretar NO (Afework et al. 1995; Marley et al. 1995). Las glándulas también reciben NO de manera paracrina, secretado por el endotelio contiguo (Torres et al. 1994). Además, el NO también se puede secretar de manera autocrina por la propia célula cromafín (Schwarz et al. 1998). El papel del NO en las células cromafines ha sido muy estudiado (Ahern et al. 2002; Cartier et al. 2001; Liu et al. 1997; Ferrero et al. 2000; Gallo & Civinini 2001; Koshimura et al. 2000; Kwan & Achike 2002; Wiesinger 2001; Marley et al. 1995; Cecilia Giulivi et al. 2006; Moncada et al. 1991).

La secreción autocrina de NO por las células cromafines se debe a las NOS. La presencia de NOS en las glándulas adrenales de mamíferos es muy diversa: en rata se ha visto inmunoreactividad tipo NOS en células glomerulosas (Natarajan et al. 1997); en especies bovinas, la actividad de la NOS se ha observado tanto en la corteza como en la médula de las glándulas; en humanos, inmunoreactividad de la NOS se ha observado en lisados celulares glomerulosos (Palacios et al. 1989).

Hasta el momento, se han realizado muchos estudios *in vitro* para averiguar el papel que juega la vía NO/cGMP en los procesos secretores de la célula cromafín, pero los resultados son controvertidos. O'Sullivan y Burgoyne (O'Sullivan & Burgoyne 1990) mostraron una potenciación en la liberación de catecolaminas inducidos por agentes liberadores de NO, aunque otros han encontrado una inhibición de la liberación de tipo dosis-dependiente (Rodriguez-Pascual et al. 1996; Oset-Gasque et al. 1994), o ningún tipo de cambio (Shono et al. 1997; Kumai et al. 1998). También parece que el NO aumenta la secreción basal de catecolaminas (Oset-Gasque et al. 1994), induciendo su síntesis a través de la activación de la tiroxina hidroxilasa (TH) (Kumai et al. 1998). Según Machado (Machado et al. 2000), el NO produce cambios en la liberación de catecolaminas modulando uno de los últimos pasos de la exocitosis, actuando por la vía sGC/PKG.

La visión clásica del tema es que el principal papel del NO es el control del flujo sanguíneo adrenal, sin embargo su papel en la regulación de la liberación de catecolaminas es pequeño (Moro et al. 1993).

8. Las células cromafines de la médula suprarrenal como modelo endocrino y neuronal.

A mediados de los años 60, Pearse y sus colaboradores (Pearse 1966) pusieron de manifiesto las grandes similitudes entre las células C del tiroides, y las células endocrinas procedentes de isletas pancreáticas. Conjeturaron que todas estas células (denominadas células APUD –amine precursor uptake and decarboxylation-) provenían de la cresta neural (Parramónet al. 1994), lo que les atribuía una característica común. Este descubrimiento dio un paso definitivo en el estudio de los sistemas nervioso y endocrino, mostrando que existían parecidos funcionales entre células secretoras de diferente localización. Posteriores investigaciones, ya en los años 70, demostraron insostenible la hipótesis de un origen común a todas las células endocrinas en la cresta neural, excepto para las células de la médula adrenal, paraganglia extradrenal y células C (DeLellis

Las células cromafines comparten con las neuronas simpáticas algunos mecanismos fundamentales: a) reciben señales eléctricas y químicas; b) reconocen y descodifican estas señales; y c) poseen la maquinaria para sintetizar, almacenar, transportar y liberar mensajeros La principal diferencia entre neuronas simpáticas y cromafines es la presencia de neuritas, pero pueden diferenciar hasta un fenotipo neuronal. En este modelo se han propuesto y estudiado procesos de exocitosis, secreción y transcripción en respuesta a estímulos, neurosecreción a tiempo real, rutas catecolaminérgicas, etc. La célula cromafín es un modelo neuronal ampliamente utilizado, funcional, homogéneo y accesible. Sin embargo, al analizar y comparar estudios hay que tener en cuenta que los cultivos de células cromafines presentan una gran heterogeneidad y diferencias entre especies.

& A. Tischler 1998). A pesar de todo, existen numerosísimas propiedades comunes a todas las células neuroendocrinas, originarias o no de los mismos precursores de la cresta neural, como canales y transportadores iónicos, componentes de la membrana y la matriz de vesículas, proteínas del citoesqueleto, etc.(Tischler 2002). De todas las células derivadas de la cresta neural, las células cromafines han sido las más estudiadas.

Las células cromafines también comparten muchos rasgos estructurales con las neuronas simpáticas. Comparten con estas neuronas algunos mecanismos fundamentales: a) reciben señales tanto de naturaleza eléctrica como química; b) reconocen y descodifican estas señales; y c) poseen la maquinaria necesaria para elaborar y generar patrones de respuesta tales como la síntesis, almacenamiento, transporte y liberación de catecolaminas y otros mensajeros (Bornstein et al. 2012).

La principal diferencia entre neuronas simpáticas y cromafines es la presencia de neuritas. En todas las especies estudiadas, las células cromafines normales aisladas de animales adultos e inmaduros, diferenciaban hasta un fenotipo neuronal en presencia de las condiciones ambientales necesarias, tanto en trasplantes, como en cultivos celulares (Unsicker et al. 1978) o *in vivo* (Aloe & Levi-Montalcini 1979). A finales de los años 70 Unsicker demostró que cultivos primarios de células cromafines derivadas de la médula adrenal, adquirían las propiedades bioquímicas y morfológicas de las neuronas simpáticas, en presencia de NGF. Un año más tarde, Aloe y Levi-Montalcini demostraron que la administración de NGF en fetos de rata era capaz de inducir la diferenciación de las células cromafines a neuronas simpáticas dentro de la glándula adrenal.

El uso de células cromafines como modelo de células neuroendocrinas emergió a mediados del siglo pasado, cuando los avances químicos hicieron posible separar la médula adrenal de la corteza. Se sabe que las células cromafines llevan a cabo procesos exocitóticos en respuesta a estímulos colinérgicos, sintetizan y almacenan catecolaminas y transportadores de membrana, expresan diferentes subunidades de receptores nicotínicos, canales de sodio, cloro, potasio...(Garcia 2002). Estas características las convirtieron, hace ya más de cuarenta años, en excelentes modelos con los que se han propuesto y estudiado procesos de exocitosis, secreción y transcripción en respuesta a estímulos, neurosecreción a tiempo real, rutas catecolaminérgicas, etc. (Cuchillo-Ibanez et al. 2002; Eiden & Hirsch 2002; Estévez et al. 1995; Parmer & Zinder 2002). La popularidad de estas células llegó a límites insospechados en 1991, cuando los Doctores Erwin Neher y Bert Sakmann recibieron el premio Nobel de Fisiología "por sus descubrimientos sobre la función de los canales iónicos únicos en las células", utilizando las células cromafines bovinas para desarrollar la técnica de patch-clamp (Hamill et al. 1981; Sakmann & Neher 1984).

Además, en contraste con otras células neuroendocrinas, las cromafines pueden ser aisladas y purificadas en grandes cantidades a partir de la médula adrenal, por lo que son fácilmente accesibles para el análisis bioquímico (Fenwick et al. 1978) y, mientras que las neuronas ejercen su actividad secretora de forma localizada alrededor del axón, las células cromafines lo hacen de forma extendida (Unsicker et al. 1997).

La función secretora de sustancias bioactivas hace que las células cromafines también sean muy útiles en el estudio y tratamiento del dolor crónico y la enfermedad de Parkinson. Las células cromafines secretan una mezcla de compuestos que poseen efectos analgésicos potentes, semejantes a los de los péptidos opiáceos. Algunos estudios en animales han mostrado que las células cromafines pueden sobrevivir y ser metabólicamente activas cuando son trasplantadas en el interior de cápsulas con membranas semipermeables en la médula espinal (Vaquero et al. 1991). Existen ensayos clínicos utilizando células encapsuladas implantadas en el espacio subaracnoideo de pacientes con dolor crónico (Buchser et al. 1996).

Todas estas similitudes y ventajas han constituido a la célula cromafín como un modelo neuronal ampliamente utilizado, funcional, homogéneo y accesible (Carmichael & Rochester 1989).

Sin embargo, al analizar y comparar las publicaciones y resultados en estudios con estas células, hay que tener en cuenta varias cosas: que los cultivos de células cromafines presentan una gran heterogeneidad, a pesar de su apariencia histológicamente uniforme; y que las células cromafines presentan diferencias entre especies. Por ejemplo, las células procedentes de médulas suprarrenales bovinas se caracterizan por tener células secretoras de epinefrina y norepinefrina separadas, mientras que las cromafines humanas no muestran esta distinción (Tischler 2002).

En nuestro grupo de investigación se han utilizado estas células para estudiar el efecto regulador de distintos neurotransmisores sobre la secreción de catecolaminas: GABA (Parramónet al. 1994), glutamato (González et al. 1998) y NO (Oset-Gasque et al. 1994). Se ha demostrado por métodos bioquímicos e inmunohistoquímicos que en las células cromafines bovinas se expresa constitutivamente la nNOS (Oset-Gasque et al. 1998). Además, también se ha observado que la presencia de la NOS está íntimamente relacionada con fibras con ACh inervando las células cromafines de rata (Holgert et al. 1995; K. Tanaka & Chiba 1996). En estas células, la ruta L-arginina/NO/cGMP tiene un importante papel inhibidor en la secreción de catecolaminas, tanto la basal como la estimulada por ACh (Schumm et al. 2002; Vicente et al. 2002) Sin embargo, al exponer estas células a altas concentraciones de donadores de NO, peroxinitritos o citoquinas, por largos periodos de tiempo,

causan su muerte por un proceso mixto de necrosis y apoptosis, en función de la concentración de NO y el tiempo de exposición (Vicente et al. 2002). Otros estudios también han mostrado la importancia de los miembros de la familia Bcl-2 en la supervivencia de éstas células (Charalampopoulos et al. 2004). Nuestros resultados indican que el tratamiento de las células cromafines bovinas con donadores de NO conlleva la formación de altas dosis de NO que provocan una muerte apoptótica de tipo dosis-dependiente, y esto se observó al analizar que la muerte apoptótica ocurría con un incremento en el número de células hipodiploides, activación de caspasa 3 y fragmentación de ADN, acompañadas de una detención del ciclo celular en la fase G0G1 y un descenso en el número de células en fases G2M y S. Además, al tratar estas células con peroxinitritos se observaba tanto apoptosis como necrosis, en función de la dosis suministrada y el tiempo de estimulación, y nos preguntamos si la intensidad del daño inicial podría estar relacionada con el mecanismo de muerte en estas células (Vicente et al. 2006).

V. Objetivos

El NO es una importante molécula señalizadora, con significativas funciones como regulador de procesos neurosecretores, procesos de plasticidad neuronal y neuroprotectores. Sin embargo, a altas concentraciones, y dependiendo de su origen celular, el NO también puede ser mediador de la muerte neuronal y de la neurodegeneración, efectos en los que están implicados sus derivados de oxidación altamente reactivos (peroxinitritos) y procesos de S-nitrosilación y nitración en tirosina de las proteínas diana. Prácticamente todas las células mamíferas son capaces de sintetizar NO de manera endógena. Esta síntesis está mediada por la familia de enzimas NOS. Cada isoforma es capaz de producir NO en un diferente rango de concentraciones. A pesar de las importantes implicaciones de esta familia, todavía queda mucho camino en su caracterización.

Trabajos previos de nuestro grupo de investigación habían demostrado el importante papel del NO, originado vía nNOS, como modulador de la secreción de catecolaminas en las células cromafines de la médula suprarrenal (Oset-Gasque et al., 1994; Oset-Gasque et al., 1998; Vicente et al., 2002; Vicente et al., 2005). Estas células, derivadas de la cresta neural, al igual que las neuronas, se han utilizado durante muchos años como un excelente modelo neuroendocrino.

En este trabajo, quisimos profundizar en el estudio de la implicación del NO, tanto exógeno como endógeno, en la neurosecreción y en la muerte celular apoptótica de las células cromafines. Caracterizamos para ello las diferentes isoformas de la NOS expresadas en estas células, y estudiamos el efecto tanto de donadores exógenos de NO, como de citoquinas y glutamato, como posibles

activadores endógenos de la NOS, sobre la muerte apoptótica de estas células. En este contexto, los objetivos que nos planteamos fueron los siguientes:

1. Conocer el efecto del NO, tanto administrado de forma exógena, como producido o inducido de forma endógena, sobre la apoptosis de las células cromafines bovinas.

2. Determinar la implicación de la vía mitocondrial en la apoptosis inducida por NO, y el papel de proteínas de la familia Bcl-2, p53 y NF-kB.

3. Estudiar el papel de las citoquinas y el glutamato como posibles generadores de NO endógeno con papel en la apoptosis.

4. Caracterizar los diferentes receptores de glutamato en las células cromafines bovinas que pudiesen estar implicados en la regulación de la neurosecreción y la apoptosis inducidas por NO endógeno.

5. Caracterizar la/s posible/s isoforma/s de la NOS implicada/s en la síntesis de NO a partir de citoquinas y glutamato (activación y expresión de nNOS y/o iNOS).

6. Evaluar la implicación del factor transcripcional NF-κB y diferentes vías de señalización celular sobre la apoptosis inducida por NO y sobre la generación de NO endógeno a partir de citoquinas y glutamato.

VI. Resultados

Mechanisms of Nitric Oxide-Induced Apoptosis in Bovine Chromaffin Cells: Role of Mitochondria and Apoptotic Proteins

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Mechanisms of Nitric Oxide-Induced Apoptosis in Bovine Chromaffin Cells: Role of Mitochondria and Apoptotic Proteins

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The aim of this work was to establish the possible involvement of mitochondria in the apoptotic event triggered by nitric oxide (NO) in chromaffin cells. Using bovine chromaffin cells in primary culture and several NO donors (SNP, SNAP, and GSNO) at apoptotic concentrations (50 μM–1 mM), we have shown that NO induces a time-dependent decrease in the mitochondrial transmembrane potential ($\Delta\Psi_m$), which correlates with the appearance of hypodiploid cells. Disruption in $\Delta\Psi_m$ is followed by cytochrome c release to the cytosol, which in turn precedes caspase 3 activation. In this mechanism participates the Bcl-2 protein family, because NO donors downregulate the expression of anti-apoptotic members of the family such as Bcl-2 and Bcl-XL, and increase the expression of pro-apoptotic members, Bax and Bcl-Xs, inductors of cytochrome c release to cytosol. Different cell signaling pathways seem to regulate Bax induction and Bcl-2 inhibition because decreased Bcl-2 levels are detected later than enhanced Bax expression. The tumour suppressor protein p53 is also upregulated in a very early phase (30 min) of the NO-induced apoptosis and may be responsible for the further induction of Bax expression. Finally, the translocation of NF-κB to the nucleus seems to be another early event in NO-induced apoptosis and it may be involved in the regulation of p53 expression. These results support strongly the participation of mitochondrial mechanisms in NO-induced apoptosis in chromaffin cells and suggest that these cells may be good models for the investigation of molecular basis of neurodegeneration and neuroprotection.
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Key words: nitric oxide; nitric oxide synthase; cell death; chromaffin cells; apoptosis; mitochondria; adrenal medulla

Nitric oxide (NO) is a signaling molecule that plays important roles in physiologic processes including smooth muscle relaxation, neurotransmission, and host defense mechanisms against tumour cells and bacteria. Endogenous NO is synthesized from L-arginine by three isoforms of NO synthase (NOS), two of which are constitutively expressed, predominantly in neurones (nNOS) and endothelial tissue (eNOS), respectively (Knowles and Moncada, 1994; Nathan and Xie, 1994; Moncada et al., 1997). Generally, constitutive NOSs release small amounts of NO and are acutely regulated by calcium/calmodulin and phosphorylation. A third isoform (iNOS) is induced during inflammation and other oxidative stress events such as hypoxia, producing large amounts of NO for long periods (Wei et al., 1995; Nathan, 1997). NO exerts its physiologic effects through the activation of guanylate cyclase (Schmidt and Walter, 1994; Denninger and Marletta, 1999) and subsequent

Abbreviations used: A, adrenaline; Ac DEVD-amc, [*N*-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin)]; Ac DEVD-CHO (CPP32), Ac-Asp-Glu-Val-Asp-CHO; Ac-Z-LEHD-CMK, Ac-Leu-Glu-His-Asp-CMK; L-Arg, L-arginine; BSA, bovine serum albumin; CA, catecholamines; CPTio, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAN, 2,3-diaminonapthalene; DMEM, Dulbecco's modified Eagle medium; cGMP, 3′-5′-cyclic guanosine monophosphate; CPP32, Caspase 3; CPTio, carboxy-PTIO; GSNO, S-nitrosoglutathion; HEPES, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethane sulfonic acid; IFNγ, gamma-interferon; IκB, nuclear factor κB inhibitor; L-NMA, *N*-methyl L-arginine; LPS, lipopolysaccharide; NA, noradrenaline; NF-κB, nuclear factor κB; NOS, nitric oxide synthase; P, peroxynitrite; P_d, deactivated peroxynitrite; PI, propidium iodide; SNAP, *S*-nitroso acetyl penicillamine; SNP, sodium nitroprusside; TNFα, tumor necrosis factor alpha; thiocitrulline, *S*-methyl-L-thiocitrulline.

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cGMP formation or through posttranslational modifications of proteins (*S*-nitrosylation and nitration) (Stamler, 1994; Jaffrey et al., 2001; Espey et al., 2002). However, the induction of a high output system for NO in response to cytokines or a massive production of NO after accumulation of excitatory neurotransmitter glutamate can result in cell death (Coyle and Puttfarcken, 1993; Lipton and Rosenberg, 1994; Bolaños et al., 1997; Eliason et al., 1997; Lee et al., 2000). Neurones (Gross and Wollin, 1995; Dawson and Dawson, 1996; Bolaños et al., 1997; Heneka et al., 1998; Gow et al., 2000; Wei et al., 2000; Figueroa et al., 2006), pancreatic β-cells (McDaniel et al., 1997), and macrophages (Messmer and Brune, 1996) seem to be particularly sensitive to NO toxicity. Whereas in some systems, NO can react with some radicals and cause cell death by necrosis, in others, the progressive intra- or extra-cellular generation of NO causes apoptosis (Fehsel et al., 1995; Palluy and Rigaud, 1996; Kröncke et al., 1997; Murphy, 1999; Gow et al., 2000; Wei et al., 2000, Figueroa et al., 2006). Several mechanisms have been proposed to explain the cellular death induced by NO (Murphy, 1999), the most common being oxidative stress production related to NO capacity to generate $ONOO^-$ (Gross and Wollin, 1995; Lin et al., 1995; Beckman and Koppenol, 1996; Pryor and Squadrito, 1995) and mitochondrial alterations (Almeida and Bolaños, 2001; Figueroa et al., 2006).

In bovine chromaffin cells, the presence of a constitutively expressed nNOS has been shown by both biochemical and immunocytochemical methods (Oset-Gasque et al., 1994, 1998; Vicente et al., 2002). In addition, the presence of NOS associated closely with ChAT-positive fibers innervating rat chromaffin cells has been reported (Holgert et al., 1995; Tanaka and Chiba, 1996). In these cells, the L-arginine/NO/cGMP pathway has an important inhibitory role in both basal and ACh-stimulated catecholamine (CA) secretion (Oset-Gasque et al., 1994; Schwarz et al., 1998; Vicente et al., 2002). However, the exposure of these cells to high concentrations of NO donors, peroxynitrite, or cytokines for a long time cause their death by a mixed necrotic and apoptotic mechanism, depending on NO concentration and time of exposure (Vicente et al., 2006).

The cell death phenomenon, besides being an important feature in the development of the nervous system, seems to be a cause for many neurodegenerative diseases such as Parkinson's disease (PD), amyotrophic lateral sclerosis, Alzheimer's disease (AD), and brain ischemia, where a gradual loss of specific sets of neurones results in disorders of movement and central nervous system (CNS) function (Thompson, 1995). Given that chromaffin cells share a common embryologic origin with neurones, the study of the effects of NO on these cells could serve as a good model to better understand the molecular mechanisms of catecholaminergic neuronal death underlying some neurodegenerative diseases.

Previous results from our laboratory indicate that treatment of adrenal chromaffin cells with either NO donors or cytokines leads to a specific and dose-dependent apoptotic death, which takes place with activation of caspase-3 and DNA fragmentation and that is accompanied by a decreased in the G2M- and S-phases of cell cycle (Vicente et al., 2006).

The molecular mechanisms of apoptosis involve several pathways among those activation of caspases, a family of cysteine proteases, represents a shared event for several pro-apoptotic stimuli. Regarding the characterization of the events upstream from caspase activation, mitochondrial damage has been reported to trigger this process. Consistent with this hypothesis, anti-apoptotic proteins such as Bcl-2 are located in the mitochondria, suggesting a role for this organelle in the induction of apoptotic death. Moreover, the release of mitochondrial proapoptotic factors, such as cytochrome c, is blocked by Bcl-2.

The aim of this study was to assess the suspected involvement of mitochondrial mediators in the apoptotic death induced by NO in chromaffin cells, emphasizing the time course of these events, and to study the role of some pro- or anti-apoptotic proteins such us Bcl-2 family, p53, and NFκB in the mechanism of NO-induced apoptosis in chromaffin cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium, FCS, HEPES, and RNAse A were from GIBCO (BRL, UK), collagenase from *Clostridium histolyticum* (EC 3.4.4.19) was supplied by Boehringer-Mannheim S.A. (Barcelona, Spain). The NOS inhibitor *S*-methyl-L-thiocitruline hydrochloride (thiocitrulline) was obtained from Tocris Cookson (Bristol, UK). Antibiotics, cytosine arabinoside, 8-fluoro-desoxiuridine (FDU), neutral red, and propidium iodide were from Sigma Chemical (Madrid, Spain). Amphotericin B was from ICN Ibérica S.A. (Barcelona, Spain). The fluorogenic substrate peptide Ac-DEVD-amc was supplied by BD Pharmingen International (Becton Dickinson, San Jose, CA). Peroxynitrite and carboxy-PTIO were purchased in Alexis Biochemicals (Lausen, Switzerland) and 2,3-diaminonaphtalene (DAN) were from Calbiochem-Novabiochem (La Jolla, CA). All other chemicals were reactive grade products from Merck (Darmstadt, Germany). Fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and tetramethyl rhodamine methyl ester (TMRM) were from Molecular Probes (Eugene, OR). Anti-Bcl-x (sc-634) and anti-caspase-9 (sc-7885) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–cytochrome c monoclonal antibody and anti-Bad (pS^{136}) were from Upstate Group Inc. (Millipore, Beverley, MA) and caspase-3 substrate Ac-DEVD-AMC, was from Pharmingen (San Diego, CA). Radiochemicals were from ICN (Irvine, CA). Other reagents were from Sigma Chemical Co. (St. Louis, MO) or Boehringer (Mannheim, Germany).

Chromaffin Cell Culture and Drug Treatments

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Oset-Gasque et al. (1994).

Cell viability was checked by Trypan blue exclusion, and chromaffin cell purity was assessed by the specific incorporation of neutral red to these cells. Both parameters were routinely >95%. Cells were suspended at a density of 0.5×10^6/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, and 40 mg/ml gentamicin), and cytostatics (10 mmol/L fluorodeoxyuridine and 10 mmol/L cytosine arabinoside), plated in 24 Costar cluster dishes and used 3–7 days after plating. After 3–7 days from plating, cells were washed three times with DMEM and exposed to NO donors adding them in concentrated form to the culture medium and mixing very gently. The cultures were then incubated during the indicated times in the figures.

Flow Cytometric Analysis of Apoptosis

Analysis of DNA content and cell cycle was carried out in a FACScan flow cytometer (Becton-Dickinson). DNA was stained with propidium iodide (PI) using the Bio-Rad reagent kit (Kinesis 50, 470-0023; Richmond, CA), following the manufacturer's protocol. Measurements were carried out using a double discriminator module to discriminate doublets. A total of 10,000 cells were acquired per sample. The percentage of cells with DNA content lower than 2C was calculated as well as the percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle, using Multicycle software (Phoenix Software, Mountain View, CA).

Measurement of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

Changes in mitochondrial transmembrane potential were measured with the fluorescent probe tetramethylrhodamine methyl ester (TMRM) as described by Tenneti et al. (1998) with some modification. TMRM is a lipophilic cation that acts as a potentiometric probe to detect changes in membrane potential, so that a reduction or an increase in fluorimetric signal means, respectively, a depolarization or a hyperpolarization of mitochondrial membrane. Chromaffin cells (2×10^6) were incubated with NO donors at different times, washed twice with PBS and loaded with 1 μM TMRM in PBS for 30 min at room temperature in the dark, and then rinsed twice. Fluorescence was measured in a FL600-BioTek spectrofluorometer (microplate reader) using filters of 530/25 nm excitation and 590/35 nm emission. Results were expressed as arbitrary fluorescence units (AFU).

Preparation of Cytosolic and Nuclear Extracts

A modified procedure based on the method of Andrews and Faller (1991) was used. Chromaffin cells (5×10^6 cells/condition) were incubated with different treatments for indicated times. Cultured cells were washed with PBS, scraped off the dishes in ice-cold PBS. Cell pellets were homogenized in 400 μl of buffer A (10 mM HEPES; pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM phenylmethyl sulfonyl fluoride, 40 μg/ml leupeptin, 2 μg/ml tosyllysyl-chloromethane, 5 mM NaF, 1 mM $NaVO_4$ and 10 mM Na_2MoO_4), and Nonidet P-40 was added to reach 0.5% (v/v). After 15 min at 4°C, the tubes were gently vortexed for 15 sec, and supernatants nuclei were collected by centrifugation at 8,000 × *g* for 15 min. The supernatants were stored at −80°C (cytosolic extracts), and the pellets were resuspended in 50 μl of buffer A supplemented with 20% (v/v) glycerol and 0.4 M KCl, then mixed for 30 min at 4°C. Nuclear proteins were obtained by centrifugation at 13,000 × *g* for 15 min, and aliquots of the supernatant (nuclear extracts) were stored at −80°C. For Western blot analysis, cytosolic and nuclear extract proteins were boiled in Laemmli sample buffer, and equal amounts of protein (15–30 μg) were separated by 10% SDS-PAGE.

Electrophoretic Mobility Shift Assays

The oligonucleotide sequence corresponding to the NFκB site was the proximal κB motive (nucleotides −92 to −65) of the rat NOS-2 promoter (Xie et al., 1993; Lowenstein et al., 1993) (tcga 5′ CCAACTGGGGACTCTCCCTTTGGGAACA 3′ and tcga 5′ TGTTCCCAAAGGGAGAGTCCCCAGTTGG 3′) was annealed with the complementary sequence by incubation for 5 min at 85°C in 10 mmol/l Tris-HCl; pH 8.0, 50 mmol/l NaCl, 10 mmol/l $MgCl_2$, 1 mmol/l DTT. Aliquots (100 ng) were end-labeled with Klenow enzyme fragment in the presence of 50 μCi of [^{32}P]dCTP and the other unlabeled dNTPs in a final volume of 50 μl. A total of 5×10^4 dpm of the DNA probe was used for each binding assay: 5 μg of nuclear protein were incubated for 15 min at 4°C with the probe and with 1 μg of poly (dI-dC), 5% glycerol, 1 mmol/l EDTA, 10 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l DTT, and 10 mmol/l Tris-HCl (pH 7.8) in a final volume of 20 μl. The DNA–protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer (Callejas et al., 2000).

Western Blot Analysis

The protein levels of IκBα, Bcl-2, Bcl-Xs, Bcl-XL, Bax, Bad-P, and β-actin were determined in cytosolic extracts. Equal amounts of protein (30–50 μg) were size-fractionated in a 10% acrylamide gel, transferred to a Hybond P membrane (Amersham, Piscataway, NJ), and, after blocking with 5% non-fat dry milk, were incubated with the corresponding antibodies and visualized by ECL as described (Vicente et al., 2002). Different exposure times were carried out with each blot to ensure the linearity of the band intensities. Band intensities were measured on a densitometric scanner (Amersham), and expressed in arbitrary intensity units.

Cytochrome c Determination

To analyze cytochrome c release, chromaffin cells (5×10^6) were resuspended in 250 mM sucrose, 25 mM Tris/HCl pH 6.8, 1 mM EDTA, 0.05% digitonin, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin. Samples were centrifuged at 13,000 × *g* for 3 min at 4°C. Supernatants were taken and considered as cytosolic fractions. The pellets (containing the mitochondrial fraction) were extracted with 40 mM HEPES pH 7.6, 0.5 M EDTA, 1 M KCl, 5% glyc-

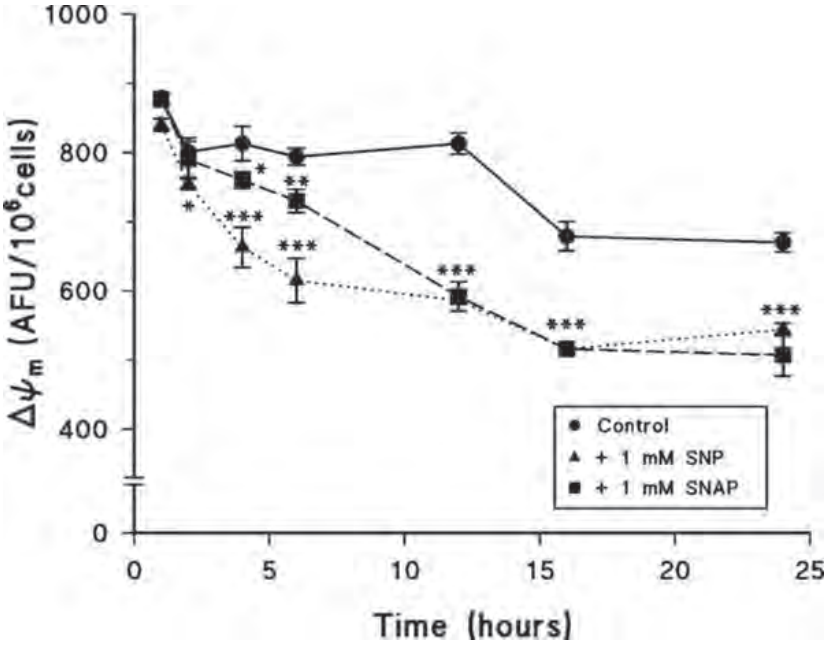

Fig. 1. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) induced by NO donors in cultured chromaffin cells. Chromaffin cells (2×10^6) were incubated in the presence or absence of NO donors, SNP and SNAP, during the indicated time periods, and loaded with 1μM of the fluorescent probe tetramethylrhodamine methyl ester (TMRM) as described in Materials and Methods. Fluorescence was measured using filters of 530/25 nm excitation and 590/35 nm emission. Results are expressed as arbitrary fluorescence units (AFU)/10^6 cells. Data are means ± SEM values obtained from three experiments, each one carried out in triplicate. Statistic compares the effect of NO donors with the control values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (one-way ANOVA test).

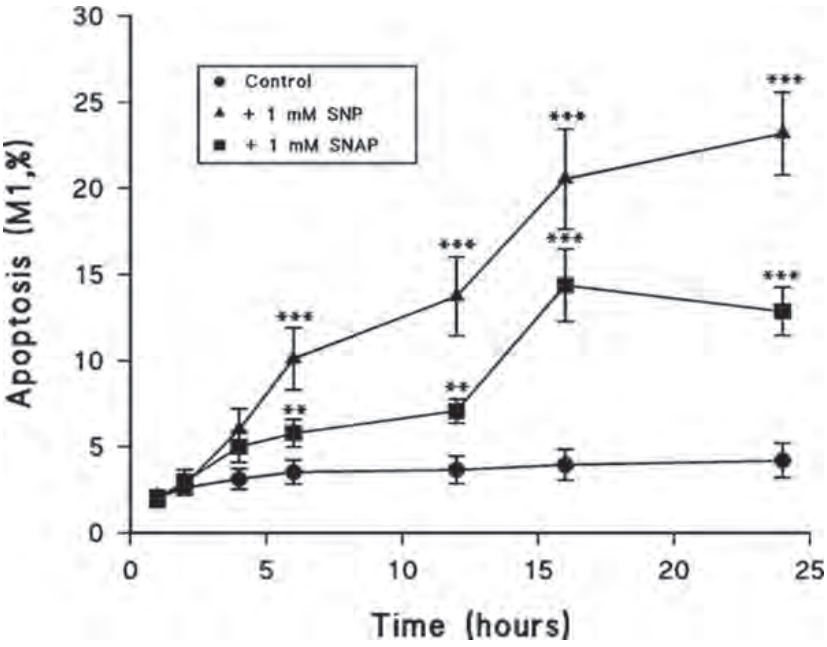

Fig. 2. NO donors induce a time-dependent increase in the number of chromaffin cells with low DNA content (apoptosis). Time course of NO donors-elicited apoptosis (1 mM each of SNP and SNAP) in cultured chromaffin cells. Chromaffin cells (10^6) were treated with 1 mM concentrations of NO donors for the indicated time periods and apoptosis was assessed by the decrease in DNA content (below 2C) (M1 fractions) measured by flow cytometry as described in Materials and Methods. Percentage of apoptosis is the mean ± SEM values obtained in three separate experiments each one carried out in duplicate. Statistic compares the effect of NO donors with the control values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (one-way ANOVA test).

erol, 0.2% Triton X-100, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin, and centrifuged at 13,000 × *g* for 3 min at 4°C. Supernatant was taken and considered as mitochondrial fraction. Cytochrome c in cytosolic and mitochondrial fractions was measured as described by Vicente et al. (2006).

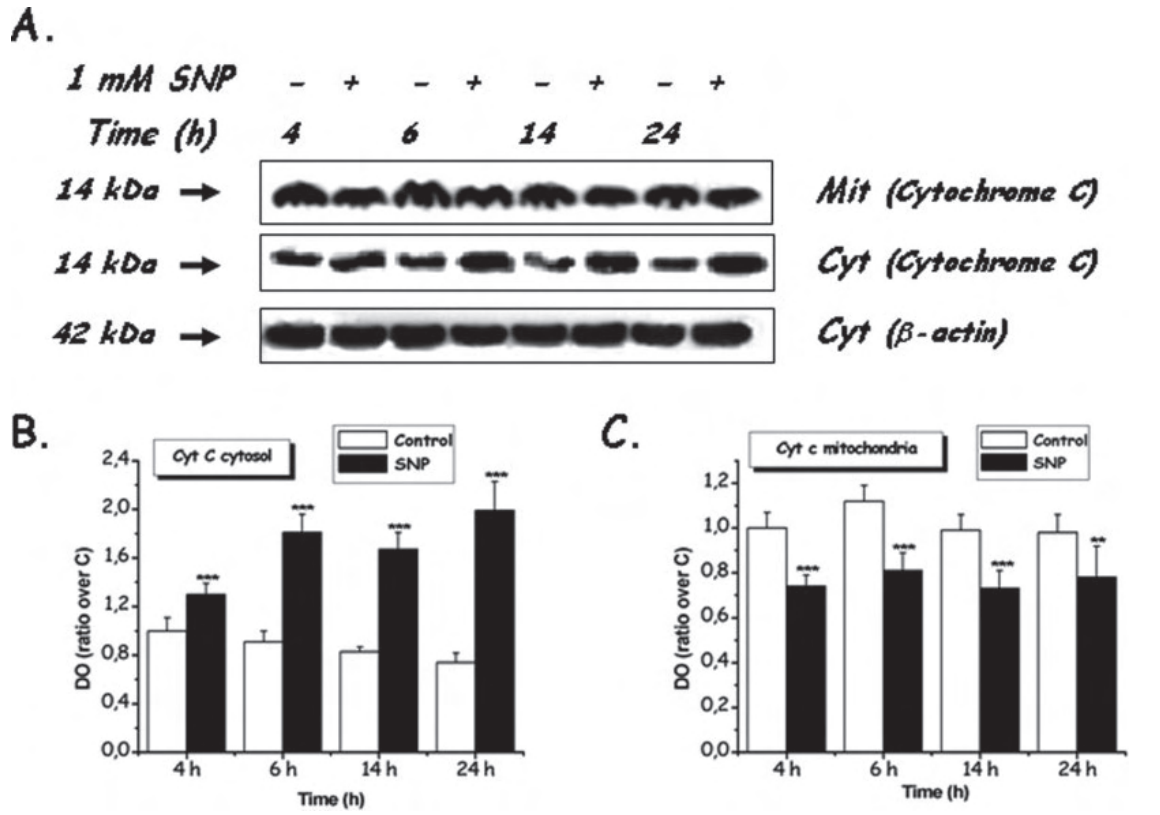

Fig. 3. Effect of NO-generating compounds on mitochondrial cytochrome c release in chromaffin cells. Chromaffin cells (5×10^6 cells/condition) were treated during the indicated time periods with 1 mM SNP. Cells were lysed, cytosolic (Cyt) and mitochondrial (Mit) fractions separated, and protein extracts from these fractions subjected to polyacrylamide gel electrophoresis and immunoblot analysis using an antibody specific for cytochrome c (Cyt C), as described in Materials and Methods. Normalization of blots was done with β-actin. **A:** Representative gels of western blot assays. **B,C:** Densitometric analysis of cytochrome c released into cytosol (B) and mitochondrial cytochrome c content (C). Data are means ± SEM from four different experiments. Statistical significances compare the effect of different treatments to the correspondent basal mitochondrial or cytosolic controls (⋆$P < 0.05$, ⋆⋆$P < 0.01$, ⋆⋆⋆$P < 0.001$) (one way ANOVA test).

Fluorimetric Analysis of Caspase 3 Activity

After 24 hr incubation of chromaffin cells (2×10^6/well) with NO donors or cytokines the culture medium containing detached cells were collected by centrifugation at 800 × *g* for 10 min at 4°C. Attached cells were scraped off in PBS and pelleted by centrifugation at 800 × *g* for 10 min. Cells were pooled and lysed at 4°C in 5 mM Tris/HCl pH 8.0; 20 mM EDTA; 0.5% Triton X-100. Lysates were clarified by centrifugation at 13,000 × *g* for 10 min. Activity of caspase 3 was measured as described by Vicente et al. (2006).

Statistics

Data were expressed as means ± SEM values of three or four independent experiments with different cell batches, each one carried out in duplicate or triplicate. Statistical comparisons were assessed by using one-way analysis of variance (ANOVA) (Scheffe's *F*-test) followed in some instance by a two-way ANOVA test. Differences were accepted as significant as $P < 0.05$ or less.

RESULTS

NO Donors Induce Depolarization of Mitochondrial Membrane Potential in Chromaffin Cells That Precede the Increase in NO-Induced Apoptosis

Because in a wide variety of cell types the induction of apoptosis is associated with a transient decrease in mitochondrial function and transmembrane potential, the first purpose of this study was to determine whether the NO-induced apoptosis in chromaffin cells was preceded by changes in mitochondrial membrane potential ($\Delta\Psi_m$). For this purpose, we use the fluorescent probe tetramethylrhodamine methyl ester (TMRM) at a concentration of 1 μM. Cells (2×10^6/condition) were treated, for 1–24 hr, with apoptotic concentrations (100 μM to 1 mM)

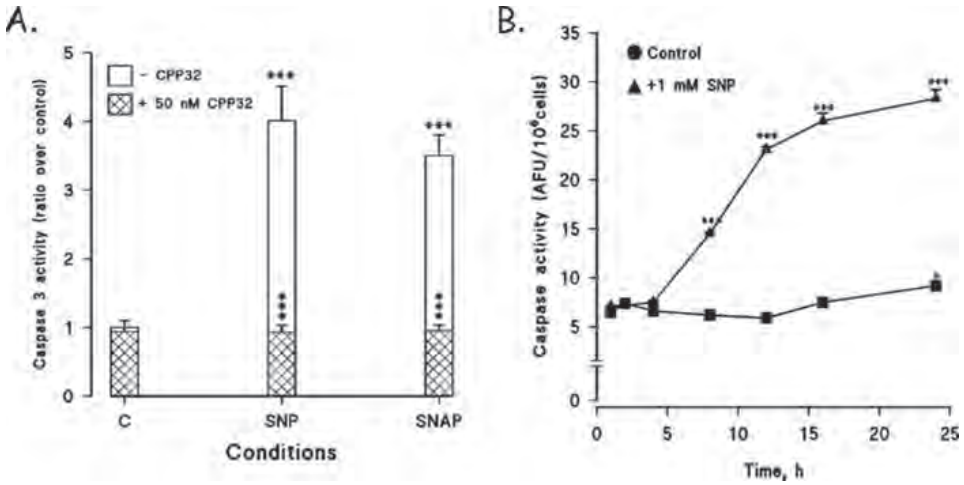

Fig. 4. Time course analysis of caspase 3 activation during NO-induced apoptosis in cultured chromaffin cells and inhibition by CPP32. **A:** Inhibitory effect of the caspase inhibitor CPP32 on increase in caspase 3 activity induced by NO donors. Cells were pre-incubated with the caspase inhibitor CPP32 at 50 nM concentration 1 hr before addition of 1 mM SNP or SNAP, and caspase activity analyzed at 16 hr. **B:** Time course analysis of caspase 3 activity. Cells were incubated with 1 mM SNP for different periods of time ranging between 1–24 hr. Cells were lysed and caspase-3 activity was assayed by using the fluorescent substrate Ac-DEVD-AMC, as described in Materials and Methods. Results are expressed as AFU/10^6 cells and are mean ± SEM of three independent experiments with duplicate dishes. Statistical significances shown in horizontal expresses the effect of NO donors on caspase 3 activity (***$P < 0.001$) and statistical significances in vertical show the inhibition by CPP32 of caspase 3 activity induced by NO donors (***$P < 0.001$) (multi variance analysis of ANOVA test).

of NO donors (SNP and SNAP) (Vicente et al., 2006), and $\Delta\Psi_m$ was measured as indicated in Material and Methods. Figure 1 shows that both NO donors induced a decrease in $\Delta\Psi_m$ in a time-dependent manner. For 1 mM SNP this effect started after 4 hr incubation with an $\Delta\Psi_m$ decrease of about 20%, reaching its maximal effect (≈30%) at 12–16 hr of incubation. In the case of SNAP, the higher decrease in $\Delta\Psi_m$ was obtained from 12 hr incubation, the effect being of similar magnitude than that obtained for SNP. These effects on $\Delta\Psi_m$ were also observed at 100 μM concentrations, but, in this case, the $\Delta\Psi_m$ began to decrease at longer times of incubation (8 hr and 16 hr for SNP and SNAP, respectively) (data not shown).

These effects on the $\Delta\Psi_m$ were correlated with the results obtained for apoptosis at the same times of incubation, apoptosis starting at 4 hr for SNP and 6 hr for SNAP, rising in a time-dependent manner and reaching a plateau between 16–24 hr (Fig. 2). These results indicate that the biggest changes in apoptosis are preceded by the biggest changes in $\Delta\Psi_m$, showing that changes in $\Delta\Psi$m precede NO-induced apoptosis.

NO-Induced Apoptosis in Chromaffin Cells Is Mediated by a Time-Dependent Increase in Cytochrome c Release and Caspase 3 Activation

In view of the above results we decided to study whether mitochondria could be implicated in the apoptotic process triggered by NO donors, checking the cytochrome c release induced by SNP and SNAP. After incubation of cells with NO donors, mitochondria were separated from cytosol and cytochrome c content in the cytosolic compartment was analyzed by Western blot analysis as described in Material and Methods. Results from Figure 3 show that cytochrome c content was decreased significantly in mitochondrial fraction from 4 hr of NO treatment, concomitant to cytochrome c cytosolic increase, reaching two to three times higher values at 6–24 hr compared to control cells.

As cytochrome c release induces caspase-9 activation and this is responsible of the caspase-3 activation we measured the activity of this enzyme in cell extracts. By using the fluorescent substrate Ac-DEVD-AMC we observed that cell incubation with SNP and SNAP at 1 mM concentration for 16 hr produced an increase in caspase 3 activity about 4-fold basal activity, respectively, which was specifically reversed by the caspase 3 inhibitor CPP32 at 50 nM concentration (Fig. 4A). A kinetic analysis of time course showed that caspase 3 activity began to increase at 8 hr of incubation with 1 mM SNP, reaching its maximal increase at later times (14–24 hr of incubation) (Fig. 4B). Maximal increases in caspase 3 activity correlates with maximal cytosolic cytochrome c release and maximal $\Delta\Psi_m$, all effects induced by NO donors and only observed under apoptotic NO donor concentrations (above 50 μM) (Vicente et al., 2006) but not lower (data not shown).

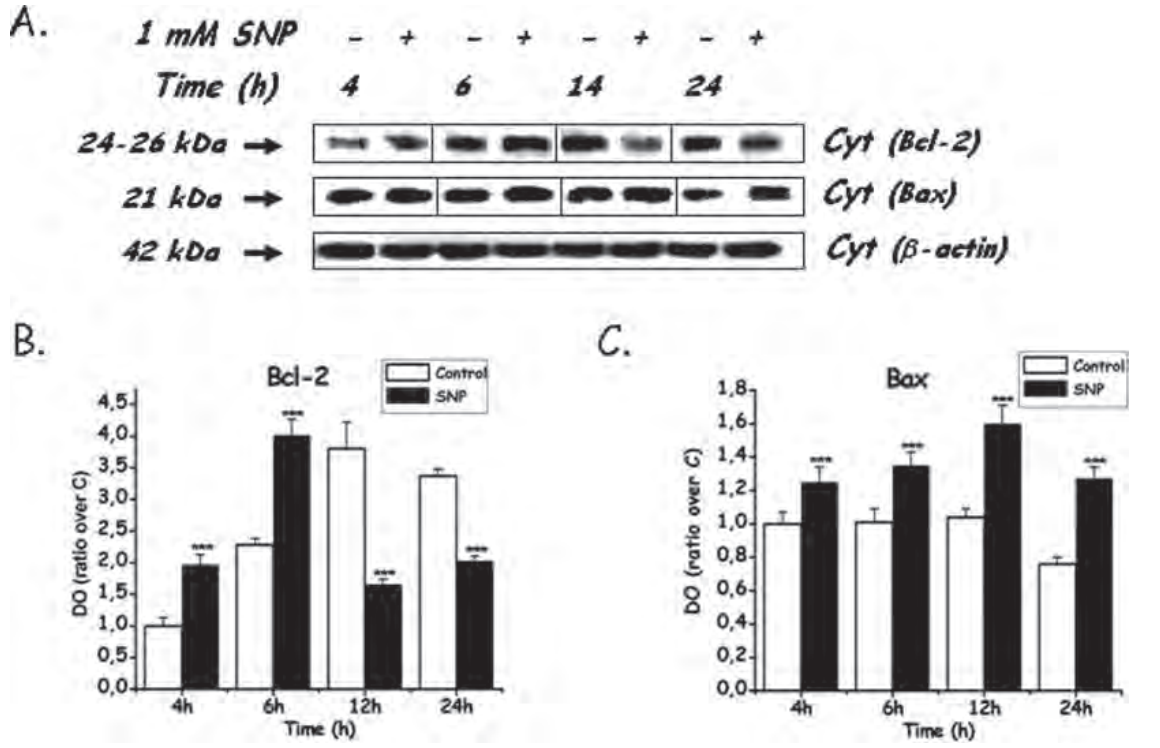

Fig. 5. Effects of NO donors on Bcl-2 and Bax proteins expression in culture chromaffin cells. Chromaffin cells (5×10^6 cells/condition) were treated for the indicated times with 1 mM SNP. Cells were lysed and protein extracts subjected to polyacrylamide gel electrophoresis and immunoblot analysis using specific antibodies for Bcl-2 and Bax proteins, as described in Materials and Methods. **A:** Western blots showing the effect of 1 mM SNP on expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins at indicated incubation times. Normalization of blots was done with β-actin. **B,C:** Densitometric analysis of Bcl-2 and Bax expressions. Data are means ± SEM from three different experiments. Statistical significances compare the effect of SNP at different with respect the correspondent basal controls (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (one-way ANOVA test).

NO-Induced Apoptosis in Chromaffin Cells Alters the Expression of Pro-Apoptotic and Anti-Apoptotic Members of the Bcl-2 Family

Because changes in proteins from Bcl-2 family are related to changes in mitochondrial events mediated by apoptosis, we checked the alterations in the expression of different members of this family with a function as antiapoptotic (Bcl-2, Bcl-XL) or apoptotic (Bax, Bcl-XS) proteins. Chromaffin cells were treated for different times with 1 mM SNP, and Bcl-2, Bcl-XL, Bax, and Bcl-XS expression were measured by Western blot.

Results from Figure 5 indicate that SNP induces Bcl-2 expression reaching a peak around 4–6 hr post-treatment, time after which Bcl-2 levels diminished. Diminution in Bcl-2 expression correlates in time with SNP-induced maximal apoptosis (Fig. 2). It was remarkable that Bcl-2 levels also increased in control cells (see below). In the case of the Bax, SNP produces a Bax increase from 4 hr, reaching the maximum increments at 14 and 24 hr, times that agrees with the maximum apoptosis values (Fig. 2). We should note that, in this case, Bax levels remained elevated for at least 24 hr, in contrast to the Bcl-2 levels that decrease after 14 hr. This indicates that the ratio Bax/Bcl-2, as apoptosis, increases with time cell incubation with SNP. Data from Table I shows that ratio Bax/Bcl-2 increases from 0.66 ± 0.05 at 4 hr incubation to 2.68 ± 0.14 at 24 hr incubation in SNP-treated cells whereas it decreases in controls at 4 hr incubation, remaining low until 24 hr incubation, due to the increase in Bcl-2 expression with incubation time (Table I).

Further on we decided to analyze the effect of other NO donors, at different concentrations, taking into account the time of maximal apoptosis observed in previous results (14 hr) over the expression of other members of the Bcl-2 family, the antiapoptotic protein Bcl-XL, and the proapoptotic Bcl-Xs. Results from Figure 6 show that: 1) all of NO donors decrease the Bcl-2

TABLE I. Effect of SNP on Bax/Bcl-2 Ratios in Bovine Chromaffin Cell Cultures at Different Incubation Times

Incubation time(hr)	Ratio Bax/Bcl-2	
	Basal	+SNP
4	1.00 ± 0.13[e]	0.66 ± 0.15[b,d]
6	0.36 ± 0.09[c]	0.92 ± 0.07[d]
14	0.26 ± 0.05[c]	3.14 ± 0.61[c,d]
24	0.29 ± 0.07[c]	2.68 ± 0.14[c,d]

Chromaffin cells (5×10^6 cells/condition) were treated for the indicated times with 1 mM SNP and protein extracts subjected to polyacrylamide gel electrophoresis and immunoblot analysis as indicated in Figure 5. Data from densitometric analysis of Bcl-2 and Bax expressions (means ± SEM from three different experiments) were used to calculate the ratios Bax/Bcl-2 in both basal and SNP treated conditions. Statistics compares the effect of incubation time on Bax/Bcl-2 ratio in both the absence or presence of 1 mM SNP in respect to basal control (15 min incubation with Locke medium).

[a]$P < 0.05$.

[b]$P < 0.01$.

[c]$P < 0.001$.

[d]Differences between the effect of the same incubation time on both cells with and without treatment with SNP at $P < 0.001$ (two-way ANOVA test for multiple variables).

[e]non significant (NS).

expression at 1 mM doses with a maximal inhibitory effect of about 30%; 2) all of them also decrease the Bcl-XL expression in a dose-dependent form, maximal inhibition being about 50%; and 3) all NO donors increase Bax and Bcl-Xs expression, these effects being dose-dependent and notably bigger in the case of Bcl-Xs (maximal effects were of about 3-fold with respect to control values whereas they were only about 1.5-fold the control values in the case of Bax).

NO-Induced Apoptosis in Chromaffin Cells Induces Bad Phosphorylation in ^{136}Ser

Bad is a member of the Bcl-2 family that promotes apoptosis by forming heterodimers with the survival protein Bcl-2, therefore preventing it from binding to Bax. Phosphorylation of Bad in ^{136}Ser by Akt/PKB is known to release Bcl-2, that increasing its antiapoptotic action (Datta et al., 1997). To confirm and have additional information on both the increase in Bcl-2 expression with time culture in control cells and the increase in Bcl-2 expression in short times SNP-treated cells, we analyzed the phosphorylation of Bad in ^{136}Ser. Bad-phosphorylation reaches a peak around 4–6 hr after SNP treatment

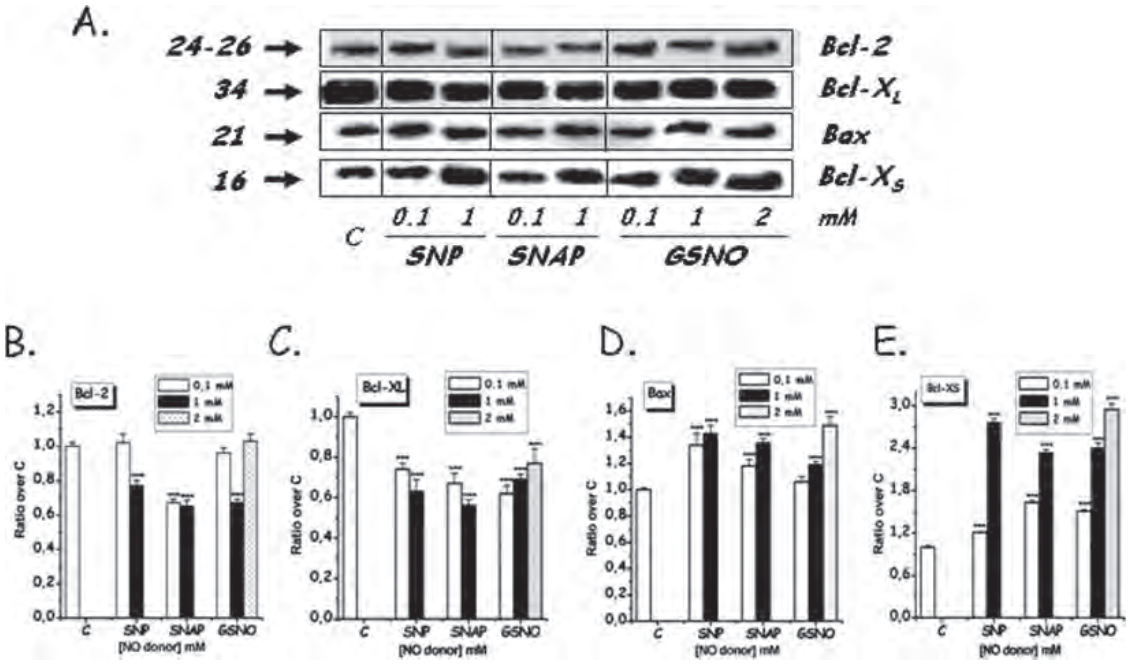

Fig. 6. Effects of NO donors on expression of different Bcl-2 family proteins involved in apoptosis. Chromaffin cells (5×10^6 cells/condition) were treated during 14 hr with the NO donors concentrations indicated. Cells were lysed and protein extracts subjected to polyacrylamide gel electrophoresis and immunoblot analysis using specific antibodies for different proteins of Bcl-2 family, as described in Materials and Methods. **A:** Representative gel of Western blot assay. **B–E**: Densitometric analysis of Bcl-2 proteins expressions. Data are means ± SEM from four different experiments. Statistical significances compare the effect of different treatments to the correspondent basal controls (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (one-way ANOVA test).

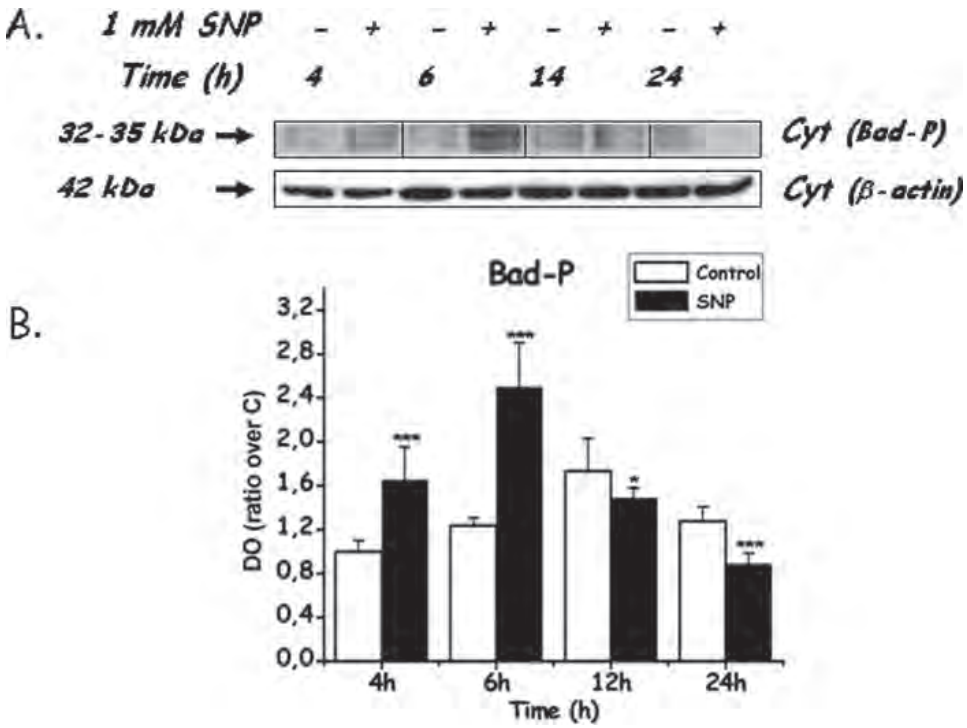

Fig. 7. Effect of NO donors on Bad phosphorylation in ^{136}Ser. Chromaffin cells (5×10^6 cells/condition) were treated for the indicated time periods with 1 mM SNP. Then, cells were lysed and protein extracts subjected to polyacrylamide gel electrophoresis and immunoblot analysis using specific antibodies for ^{136}Ser P-Bad, as described in Materials and Methods. **A:** Representative western blot showing the effect of 1 mM SNP on BAD phosphorylation in ^{136}Ser at indicated incubation times. Normalization of blots was done with β-actin. **B:** Densitometric analysis of BAD phosphorylation. Data are means ± SEM from four different experiments. Statistical significances compare the effect of different treatments to the correspondent basal controls (★$P < 0.05$, ★★$P < 0.01$, ★★★$P < 0.001$) (one-way ANOVA test).

diminishing afterwards, quite a similar effect to that observed for Bc12 expression (Fig. 7). Moreover, P-Bad is also increased in control cells, although in smaller proportion than Bcl-2.

NO Donors Increase p53 Expression

Protein p53 determines cell fate in either recovery or apoptosis. To know whether p53 is involved in NO donor-induced apoptosis we examined the protein expression levels at different times of cell incubations with SNP or GSNO. Data from Figure 8 show that both NO donors at 1 mM concentration induced a very rapid increase of expression levels of this protein in whole cell extracts in a time-dependent way (Fig. 8A,B). Both NO donors increase p53 levels as early as 15 min of incubation, reaching a peak of expression around 1 hr and keeping levels above control values for at least 24 hr (results not shown). This NO-donor-induced increase in p53 expression levels was also shown in nuclear extracts. In Figure 8C,D we observed that maximal SNP-induced p53 levels increases in nuclear extracts at 1 hr to lower at longer times.

Involvement of NFκB and IκBα in NO Donor-Induced Apoptosis in Chromaffin Cells

NFκB is a transcription factor that in basal conditions is found bound to IκB in the cytosol. When IκB is phosphorylated, NFκB is released from this complex, and migrates to the nucleus where it induces the synthesis of several proteins, p53 among them. Because the NO donors used in our experiments induce an increase in p53 expression, we investigated the possibility of a mechanism of transcription of this protein mediated by NFκB. Chromaffin cells were treated at different times (15 min to 24 hr) with 1 mM of each donor and cytosolic IκBα levels and NFκB-binding to DNA were measured by Western blot analysis and EMSA, respectively.

Data from Figure 9 indicate that SNP and GSNO induce NFκB translocation to the nucleus at very short times of 15–30 min (Fig. 9A) to decrease at later times. In all cases, two bands appeared corresponding to p65/p50 (up), p50/p50 (down) dimmers, being both bands and in special that of homodimers p50 which shown the biggest changes. The NFκB activation induced by NO

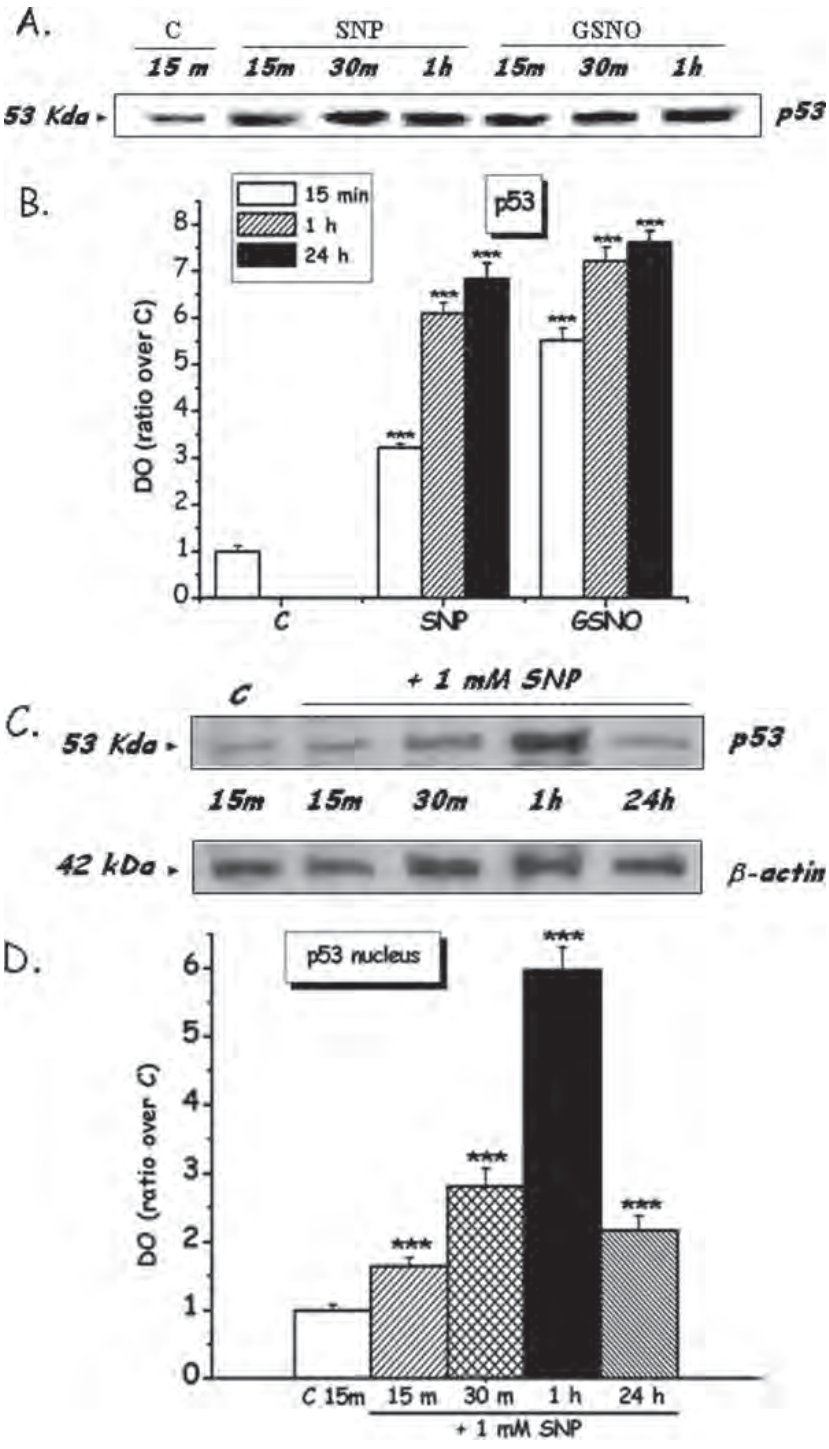

Fig. 8. Time-dependent activation of endogenous p53 expression by NO donors in cultured chromaffin cells. Whole-cell (**A,B**) or nuclear (**C,D**) extracts of bovine chromaffin cells treated with or without 1 mM SNP or 1 mM GSNO (A,B) or 1 mM SNP (C,D) for the indicated time periods were subjected to Western blotting technique and probed with p53 antibody as indicated in Materials and Methods. A–C: Representative gels of Western blot assay for p53. B–D: Densitometric analysis of p53 expression in whole cell (B) or nuclear (D) extracts. Data are means ± SEM from three different experiments. Statistical significances compare the effect of different treatments to the correspondent basal control (⋆$P < 0.05$, ⋆⋆$P < 0.01$, ⋆⋆⋆$P < 0.001$) (one-way ANOVA test).

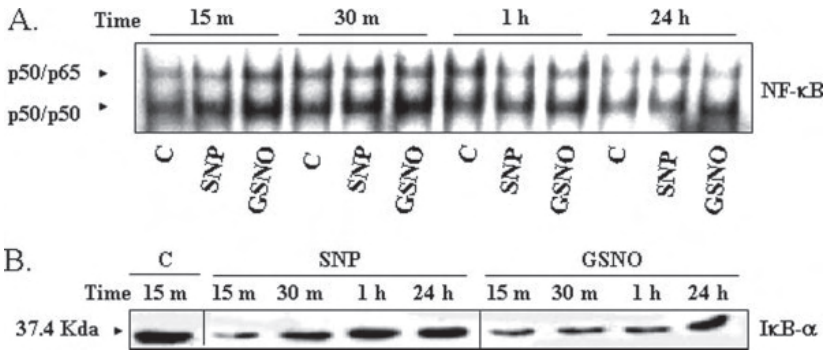

Fig. 9. Effects of NO donors on NFκB binding to DNA and IκBα degradation in cultured chromaffin cells. Chromaffin cells (5×10^6) were incubated in the absence or presence of 1 mM SNP or 1 mM GSNO during the indicated time periods. Cells were lysed and cytosolic and nuclear extracts were obtained as indicated in Materials and Methods. **A:** Total IκB-α was determined in cytosolic extracts by western blot techniques using a specific antibody. **B:** NF-κB activity was assessed by measuring the NF-κB binding to DNA (iNOS promoter) in nuclear extracts by EMSA techniques. Results show a representative Western blot assay (A) or EMSA assay (B) from four experiments.

donors was indirectly assessed by evaluating IκBα degradation in the cytosol (Fig. 9B). This effect begins at short times of incubation with NO donors (15 min) and recovers progressively at longer times until a total recovering at 24 hr. Controls did not suffer any change at any incubation time. These results show that IκBα degradation and NFκB activation are cyclic processes and that both events are related closely.

DISCUSSION

The involvement of mitochondrial mediators and different apoptotic proteins in the NO-induced apoptotic death in chromaffin cells was assessed emphasizing the time course of these events. Using bovine chromaffin cells in primary culture and different NO donors (SNP, SNAP, and GSNO) at apoptotic concentrations ranging 100–1000 μM (Vicente et al., 2006), we have shown that NO induces a time-dependent decrease in transmitochondrial membrane potential ($\Delta\Psi_m$). This effect was detected after 4 hr of NO donor incubations, although maximal inhibitions were obtained at times between 12–16 hr incubation, paralleling the NO concentration reached in the cells (Ramamurthi and Lewis, 1997; Schmidt et al., 1997; Ferrero et al., 1999). The disruption in $\Delta\Psi_m$ was correlated with apoptosis and with caspase-3 activation, which was reversed by the caspase-3 inhibitor CPP32 indicating that, in chromaffin cells, the apoptosis mediated by this donor occurs by the caspase activation pathway. This caspase pathway is activated via mitochondrial alterations and not via Fas receptor, as Martin et al. (2005) found using motor neurons, because in chromaffin cells the NO donors used induce cytochrome c release that in turn precedes caspase 3 activation. Similar results were found by Figueroa et al. (2006) in cortical neurons. These authors found that in cortical neurones, SNAP induced apoptosis, mediated by caspase-9, caspase-3 activation, and release of cytochrome c. In chromaffin cells, cytochrome c release preceded both NO donor-induced activation of caspase-3 and appearance of hypodiploid cells measured by flow cytometry, events that occurred after 8 hr of treatment and were maximal after 24 hr. Both events occurred upstream from the caspase 3 activation and subsequent apoptosis in chromaffin cells.

The involvement of the Bcl-2 protein family in the NO-induced apoptosis in chromaffin cells was shown by evaluating the effect of NO donors on the expression of different antiapoptotic (Bcl-2 and Bcl-XL) and proapoptotic (Bax and Bcl-Xs) members of this family. Our results show that at short times (4–6 hr) of SNP incubation there was an increase in both Bcl-2 and Bax levels. This Bcl-2 increase could indicate the activation of a survival pathway as an attempt to protect chromaffin cells against NO-induced apoptosis. At this time, however, we also measured apoptosis by flow cytometry, caspase-3 activation, and cytochrome c release, all of them indicatives of apoptosis, data that seem to disagree with an elevated Bcl-2 expression. This apparent disagreement could be explained if we take into account the idea that apoptosis is not depending on the increase or decrease of Bcl-2 or Bax proteins separately but on the ratio between these two proteins. Harris and Thomson (2000) and Chae et al. (2004) found that in rat vascular smooth muscle, NO-induced apoptosis involves an increase in the ratio of Bax/Bcl-2 gene expression, which leads to the release of cytochrome c from the mitochondria to the cytosol. In chromaffin cells there was a decrease of Bcl-2 expression after 14–16 hr of incubation preceded by a time-dependent increase in the expression of this protein, thus indicating that the initial activation of a survival pathway as an attempt to protect chromaffin cells against NO-induced apoptosis could not be maintained at longer times of incubation. However, at all time periods studied, Bax expression was still

increased, indicating that the ratio Bax/Bcl-2 increases with time (from about 0.6 at 4 hr incubation to about 3 times at 14–24 hr incubation; Table I). This is correlated with the highest cytochrome c release found at these times. Other results confirming the implication of pro-apoptotic Bcl-2 proteins in the apoptotic death induced by NO donors in chromaffin cells are the fact that these compounds decrease the levels of the antiapoptotic protein Bcl-XL and increase the expression of the apoptotic protein Bcl-Xs in a dose-dependent way. The involvement of Bcl-2 and Bcl-XL in apoptotic processes has been evidenced by several authors in different cell types (Allen et al., 1998; Banasiak et al., 2000; Glasgow and Perez-Polo, 2000; Hu et al., 2003). In chromaffin cells the most significant effect was the dose-dependent induction of the pro-apoptotic protein Bcl-Xs expression, which was maximal between 14–16 hr of incubation with the NO donors. Because main changes in Bax and Bcl-Xs expression preceded the decrease in Bcl-2, it could be possible that at short times Bcl-2 induction is regulated by different cell signaling mechanisms than Bax induction.

To have additional evidence on the survival pathway activated in basal and short-times-SNP treated cells, we analyzed the phosphorylation of Bad. Bad is a member of the Bcl-2 family, which promotes apoptosis by forming heterodimers with the survival protein Bcl-2, thus preventing it from binding to Bax. We assayed phosphorylation in ^{136}Ser because Akt/PKB phosphorylation of Bad in serine 136 leads to the release of Bcl-2, that increasing its antiapoptotic action (Datta et al., 1997). Our data indicate that in chromaffin cells the effect of SNP on the time course of Bad phosphorylation is quite similar to that observed for Bcl-2 expression. That is, phosphorylation of Bad reaches a peak around 4–6 hr after SNP-treatment, diminishing afterwards. Moreover, P-Bad also increases in control cells although in smaller proportion than Bcl-2. These data seem to indicate that, in basal conditions, maybe mediated by the presence of survival factors in the medium, or when chromaffin cells are subjected to an insult like high concentrations or long exposures to NO, a survival pathway, involving Bad phosphorylation and Bcl-2, could be activated. This survival pathway could induce Bad phosphorylation, leading to the dissociation of Bad from pro-survival Bcl-2 proteins, and their subsequent association with Bax, or other apoptotic proteins, to inhibit apoptosis. These effects could be mediated by growth-factor-induced activation of PI3K/Akt or other survival kinases. In fact, in chromaffin cells we observe that NGF and insulin increase Bcl-2 expression and Bad phosphorylation while inhibiting Bax expression. Our results support a role for Bcl-2 and Bad phosphorylation in the mechanism by which survival kinases or other survival signals protect chromaffin cells against apoptosis, induced by light or middle apoptotic stimuli (i.e., age of culture, medium change stress, short high NO exposures). As the results of Datta et al. (2002) indicate, Bad phosphorylation could attenuate death signaling pathways by raising the threshold at which mitochondria release cytochrome c to induce cell death.

The precise mechanism of how NO induces changes in Bax and Bcl-2 gene expression is unknown. It is known that in some cases (as in tumoral cells) cytotoxic effects of NO and peroxynitrite-induced DNA damage is mediated by p53 accumulation. However, in other cellular systems NO can induce cell death by p53-independent mechanisms (Messmer and Brune, 1996). Some investigators have focused on p53 as a linkage between NO and Bax/Bcl-2 genes, because NO is known to induce the accumulation of p53 (Brune et al., 1998; Lopez-Farré et al., 1998) that is a direct transcriptional activator of the Bax gene (Miyashita and Reed, 1995) and a transcriptional inhibitor of the Bcl-2 gene by interacting with Brn-3a in macrophages (Budhram-Mahadeo et al., 1999). However, in other tissues like vascular smooth muscle, Kibbe et al. (2002) showed that p53 may protect these cells from NO-mediated apoptosis, in part through regulation of the MAPK pathway.

In our study, one of the earlier events associated with NO-dependent apoptosis is a rapid rise and accumulation of the tumor suppressor protein p53. In chromaffin cells, the induction of the expression of p53 occurred at 15 min of incubation with different NO donors, and the effect was time-dependent and maximal between 14–24 hr. The NO-dependent increase of p53 has been consistently observed and it has been suggested that this accumulation leads to the expression of several proteins, which ultimately participate in apoptosis (e.g., Bax). After p53 upregulation, Bax levels could increase and heterodimerize with other members of the Bcl-2 family, thus triggering apoptosis. Indeed, as in chromaffin cells, in other cell types it has been observed that the overexpression of Bcl-2 inhibits p53-dependent apoptosis. All this suggests a role for p53 as an initial step in the NO-dependent apoptotic process in chromaffin cells. These results agree with those from Gómez-Lázaro et al. (2005) showing the contribution of a transitory increase in p53 expression to veratridine-induced apoptosis in chromaffin cells. Both results support the importance of p53 as a downstream effector of apoptosis involving mitochondrial participation pathways.

Some studies have focused on the involvement of nuclear factor-NFκB as a mediator of apoptosis (Ibe et al., 2001). In those studies, investigators showed that SNP decreased NFκB binding activity in human vascular smooth muscle cells. In chromaffin cells we found that the activation of NFκB, measured by the binding of NFκB to nuclear proteins and Iκ-Bα degradation, occurs after 15–30 min of incubation with NO donors and cytokines, and seems to be another early mechanism involved in NO-induced apoptosis in these cells. Thus, in NO donor-induced apoptosis in chromaffin cells, NFκB could be a death factor. In fact, 10 μM SN50 (an inhibitor of NFκB translocation) increased the SNP-induced apoptosis in chromaffin cells by about 40%, despite the fact that Bax expression and cytochrome c release were inhibited. Therefore, despite its

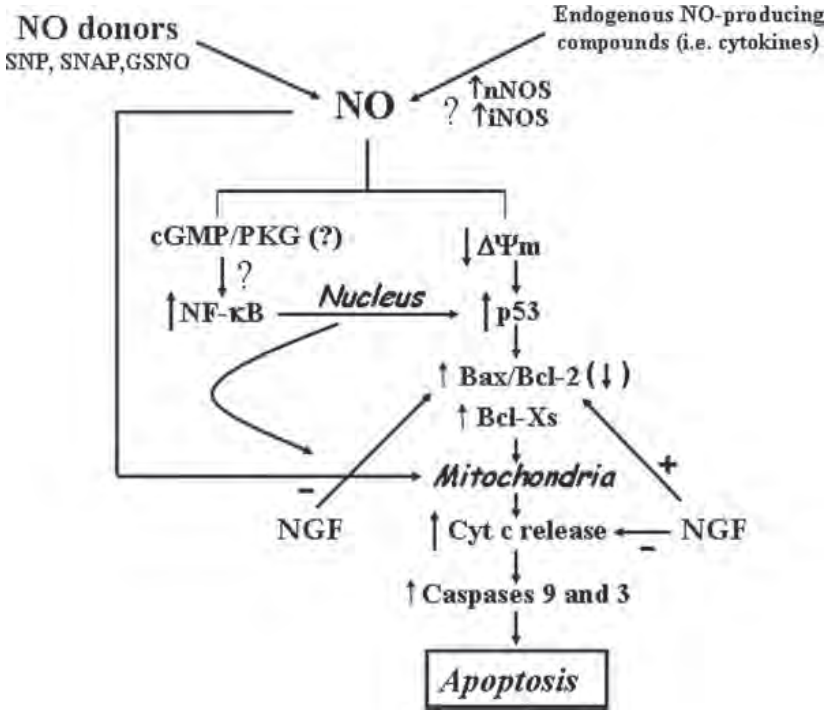

Fig. 10. Summary of the proposed mechanism for the apoptotic events induced by NO in primary cultures of bovine chromaffin cells. NO, which could be generated in the cells by induction of nNOS or iNOS by cytokines or activation of glutamate receptors (Vicente et al., 2002, 2006), mediates a decrease in $\Delta\Psi_m$, which is followed by a decrease in the Bcl-2 expression, an increase in cytochrome c release into the cytosol, the activation of caspase 9 and 3, and apoptotic cell death. It is likely that the induction of p53 expression is one of the earliest events after the initial insult, which could be responsible for the regulation of the expression of Bcl-2 family proteins. The expression of p53 could be, in turn, regulated by NFκB. NGF is able to block these events, thus preventing both caspase activation and cell death (Vicente et al., 2006).

activation, NFκB is not able to rescue cells from NO-induced apoptosis but is a mediator of NO donors-induced chromaffin cell death.

Figure 10 summarizes the molecular mechanism proposed for the involvement of mitochondrial mediators in NO-induced apoptosis in chromaffin cells. NO donors mediate a decrease in ΔΨm, followed by a decrease in Bcl-2 expression (maybe linked to Bad phosphorylation), an increase in cytochrome c (Apaf 2) release into the cytosol, the activation of caspase 9 and 3 and apoptotic cell death. It is likely that the induction of p53 expression is one of the earliest events after the initial insult, and could be responsible for the regulation of the expression of the Bcl-2 family proteins. The expression of p53 could be, in turn, regulated by NFκB. The neurotrophin NGF is able to block these events, thus preventing both caspase activation and cell death (Vicente et al., 2006). The participation of the cGMP/PKG pathway or protein *S*-nitrosylation in this mechanism, as well as its regulation by specific transduction signal pathways or neuroprotectors, remain to be investigated.

These results support strongly the role of mitochondrial mediators in NO-induced apoptosis in chromaffin cells and point out these cells as a good model for investigating the molecular mechanisms involved in neurodegenerative diseases underlying catecholaminergic neuronal death and the mechanisms of neuroprotection against apoptotic death underlying these important diseases.

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Signaling mechanisms of interferon gamma induced apoptosis in chromaffin cells: involvement of nNOS, iNOS, and NFκB

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Abstract

Previous work of our group stated that exogenously added and endogenous nitric oxide (NO) generated by cytokines induce apoptosis in chromaffin cells. In this work, we investigate the specific regulation of the NO synthase (NOS) isoforms, inducible NOS (iNOS) and neuronal NOS (nNOS), and their particular participation in cell death induced by interferon gamma (IFNγ). Lipopolysaccharide (LPS) and IFNγ increase iNOS expression, with no effect on nNOS expression. On the other hand, dexamethasone increases basal nNOS expression but decreases LPS + IFNγ-induced iNOS expression. IFNγ-induced cell death was abolished by W-1400, a specific iNOS inhibitor, but only partially by nNOS inhibitors [*N*-ω-propyl-L-arginine (*N*-PLA), 3-Bromo-7-nitroindazol (7-NI), L-methyl thiocitrulline and *N*-methyl L-arginine], indicating the main iNOS participation in chromaffin cell death. IFNγ and LPS induce nuclear factor κB (NFκB) translocation to the nucleus, a process implicated in activation of iNOS expression, as inhibition of NFκB translocation, by SN50, decreased iNOS expression. In addition, IFNγ and LPS induce ^{847}Ser-nNOS phosphorylation, inhibiting nNOS activity. Both processes, nNOS phosphorylation and iNOS expression induced by LPS + IFNγ, are regulated by Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, as IFNγ increases ^{727}STAT-3 phosphorylation and specific inhibitors of JAK/STAT pathway, such as AG490, inhibited both processes. Taken together, these results support the hypothesis of an inactivating phosphorylation of nNOS by IFNγ, via JAK/STAT, in bovine chromaffin cells. Low NO concentrations achieved by this event, would activate NFκB translocation, increasing iNOS expression and generating, this last, high apoptotic NO concentrations.

Keywords: apoptosis, cell death, chromaffin cells, JAK/STAT, nuclear factor κB, nitric oxide synthase, signal transduction.

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The free radical nitric oxide (NO) is a cellular messenger playing very important roles in physiological processes including regulation of vascular tone, neuronal transmission, and modulation of immunological and inflammatory reactions as well as cellular growth, survival, apoptosis, proliferation, and differentiation (see Madhusoodanan and Murad 2007). In the nervous system, NO has a very important function as a regulator of neurotransmission, synapse formation, synaptic plasticity, and brain development and also in pathological events of neuronal cell death underlying neurotoxicity and neurodegeneration [see Calabrese *et al.* (2007) for a review].

Endogenous NO is synthesized from L-arginine by three isoforms of NO synthase (NOS). Two isoforms are expressed constitutively, the neuronal (nNOS or type 1) and the endothelial (eNOS or type 3) and one inducible under pathological conditions and inflammation (iNOS or type 2), producing large amounts of NO for up to long periods (Bredt 1999). All three isoforms are found in the CNS. The induction of a high output system for NO in response to cytokines (Liu *et al.* 2002) or a massive

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Abbreviations used: 7-NI, 3-Bromo-7-nitroindazole; cGMP, 3′-5′-cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; IFNγ, interferon gamma; iNOS, inducible nitric oxide synthase; IκB, nuclear factor κB inhibitor; JAKs, Janus Kinases; L-NMA, *N*-methyl L-arginine; LPS, lipopolysaccharide; MEK, Mitogen Activated Protein Kinase Kinase; NFκB, nuclear factor κB; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; *N*-PLA, *N*-ω-propyl-L-arginine; PKA, cAMP-dependent protein kinase; STATs, Signal Transducers and Activators of Transcription; TNFα, Tumor necrosis factor α; W-1400, *N*-(3-aminomethyl)benzyl) acetamidine.

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production of NO following accumulation of glutamate (Nakamura *et al.* 2007) can result in cell death and in pathological events underlying neurotoxicity and neurodegeneration. In fact, inhibition of nNOS and iNOS activity ameliorates the progression of disease pathology in animal models of different neurodegenerative diseases [see Calabrese *et al.* (2007) for a review].

Apoptosis is recognized as a normal feature in the development of the nervous system and may also play a role in neurodegenerative diseases, excitotoxicity and aging (Sastry and Subba 2000). NO plays a dual role in apoptosis, being both pro and anti-apoptotic (Boyd and Cadenas 2002; Calabrese *et al.* 2007). As a pro-apoptotic factor, it has been proved that NO generates peroxynitrite, activates death receptors, inhibits mitochondrial ATP synthesis, and inactivates antioxidant enzymes; on the other hand, NO can act as an anti-apoptotic signal, stimulating 3′-5′-cyclic guanosine monophosphate (cGMP) production, or protein *S*-nitrosylation (Kim *et al.* 2001). These roles much depend on the concentration of NO: nNOS produces small amounts of NO that may become deleterious when stimulated for long periods of time, whereas, once activated, iNOS produce high amounts of NO for long periods of time, actually as long as the enzyme remains activated. This high output of NO could get deleterious to the surrounding cells.

The pathways involving NO reactivity are countless. Among them, major effects are due to nuclear factor κB (NFκB) and STAT3. These proteins have been proven to mediate the effects of cytokines on inflammation, translocating to the nucleus where they activate the transcription of the iNOS, and therefore controlling the amount of this enzyme (Kleinert *et al.* 2004).

In bovine chromaffin cells, the presence of a constitutively expressed nNOS has been demonstrated (Vicente *et al.* 2002). In these cells, the L-arginine/NO/cGMP pathway has an important inhibitory role in both basal and acetylcholine-stimulated catecholamine secretion (Oset-Gasque *et al.* 1994; Vicente *et al.* 2002). Moreover, previous results from our group indicate that the exposure of these cells to high concentrations of NO donors, peroxynitrite, or cytokines for long periods induced apoptosis in chromaffin cells (Vicente *et al.* 2006; Pérez-Rodríguez *et al.* 2007). However, the intracellular origin of endogenous NO generated by cytokine-treatment of chromaffin cells is unknown, as different NOS isoforms could be implicated. Thus, in this work we try to asses the role of intracellular NO in chromaffin cell apoptotic death by studying (i) the presence and specific regulation of different NOS isoforms in chromaffin cells and (ii) their specific participation in interferon gamma (IFNγ)-induced apoptosis on these cells. Given the importance of transcriptional factor NFκB as regulator of both cell death/survival process and transcriptional activation of iNOS, we also study the role of this transcription factor on both activation of different NOS isoforms and NO-induced apoptosis. Finally, we assess the possible involvement of the JAK/STAT pathway on these effects.

Materials and methods

Chromaffin cell culture and drug treatments

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Vicente *et al.* (2006) with some modifications. Briefly, glands were incubated with Ca^{2+}-free Locke containing 0.1% protease (Sigma, St Louis, MO, USA) for 20 min (2 × 10 min). Medulla was detached from cortex, submitted to mechanical disgregation, and further incubated with 0.1% collagenase (Worthington) in Ca^{2+}-free Locke for 25 min with shaking. After digestion, solution was filtered through a 190 µm pore nylon membrane, and chromaffin cells purified through a series of 10 min centrifugations (1 × 180 *g*, 6 × 50 *g*) using a 4% albumin gradient in the last one. Cell viability and purity checking and cell plating and treatments were performed as described (Vicente *et al.* 2006).

Measurement of nitrite production

Nitrites were determined with the spectrofluorimetric method of Misko *et al.* (1993), with minor modifications as described by Vicente *et al.* (2006). This method is based on the measurement of the fluorescent product 1-(H)-naphthotriazole formed by the reaction of nitrites with 2,3-diaminonaphtalene in acidic conditions. Samples were calibrated with a standard curve of freshly prepared nitrites and results were expressed as arbitrary fluorescence units.

Measurement of NOS activity (citrulline assay)

Cultured bovine chromaffin cells (10^6/condition) were incubated in the absence or presence of 200 ng/mL IFNγ and/or in the absence or presence of different NOS inhibitors, for 24 h either in a Dulbecco's modified Eagle's medium without phenol red containing 2.5 mM Cl_2Ca (total activity) or in a medium without calcium containing 4 mM EGTA + 10 µM calmidazolium (1-[bis(4-chlorophenyl) methyl]-3-[2-(2;4-dichlorophenyl)-2-{2;4-dichlorobenzyloxy}ethyl]-1H-imidazolium chloride) (calcium-independent activity). Then, supernatants were taken out for nitrite measurements, cells were lysed with 400 µL of pure water and NOS activity was measured by quantification the production of [U-^{14}C]L-citrulline from [U-^{14}C]L-arginine as described (Vicente *et al.* 2002). Calcium-dependent NOS activity was calculated as the difference between the amount of synthesized [^{14}C]L-citrulline in the presence of physiological extracellular calcium and that formed in the absence of extracellular calcium.

Measurement of cell viability by the XTT test

Changes in cell viability induced by IFNγ, in the presence and absence of NOS inhibitors, was measured by the Cell Proliferation Kit II (Hoffmann-La Roche Ltd Diagnostics, Basel, Switzerland) (XTT assay) as described (Figueroa *et al.* 2005). Results were expressed as ratios over the respective controls (not-treated cells).

Flow cytometric analysis of apoptosis

Measurement of apoptosis induced by IFNγ, in the presence and absence of NOS inhibitors, was carried out by Flow cytometry as

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described (Figueroa *et al.* 2005). Results were expressed as ratios over the respective controls (not-treated cells).

Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts were isolated by a modified procedure based on the method of Andrews and Faller (1991) as previously described (Pérez-Rodríguez *et al.* 2007). Aliquots of extracts were analyzed for protein content using the Bio-Rad (Hercules, CA, USA) protein reagent.

Electrophoretic mobility shift assays (EMSA)

The oligonucleotide sequence corresponding to the NFκB site was the proximal κB motive (nucleotides −92 to −65) of the rat NOS-2 promoter (tcga 5′-CCAACTGGGGACTCTCCCTTTGGGAACA-3′ and tcga 5′-TGTTCCCAAAGGGAGAGTCCCCAGTTGG-3′). electrophoretic mobility shift assays (EMSA) assays were performed as described (Pérez-Rodríguez *et al.* 2007).

Western blot analysis

Western blots were carried out as described by Pérez-Rodríguez *et al.* (2007). Band intensities were measured on a densitometric scanner, and normalized with respect to β-actin expression.

RT–PCR analysis

RNeasy Mini Kit (Quiagen Ltd, Sussex, UK) was used for total RNA isolation. RT was carried out for 1 h at 55°C with oligodeoxythymidylate primer using 5 μg of total RNA from each sample for complementary DNA synthesis.

Semiquantitative and real-time quantitative PCR to determine the levels of rat NOS and housekeeping Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs was performed by using the following specific primers synthesized at Sigma-Genosys.

Primer	Sequence
GA3PDH forward	5′-CACAGTCAAGGCAGAGAACG-3′
GA3PDH reverse	5′-TACTCAGCACCAGCATCACC-3′
nNOS forward	5′-TGATCATCTCTGACCTGATTCG-3′
nNOS reverse	5′-ATAGCTCAGGTCCACCAAGG-3′
eNOS forward	5′-GCGATGTCACTATGGCAACC-3′
eNOS reverse	5′-CGTGATAGCGTTGCTGATCC-3′
iNOS forward	5′-AGCATGTGTTCACCATGAGG-3′
iNOS reverse	5′-GCAGGTAGCTTGGAGCTTGG-3′

Real-time PCR

The SYBR Green PCR Master Mix and the 7900 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) were used to detect the real-time quantitative PCR products of reverse-transcribed cDNA samples, according to the manufacturer's instructions. q-PCR conditions were: 95°C (10 min) followed by 40 cycles of 15 s at 95°C and annealing for 1 min at 60°C. Three independent quantitative PCR assays were performed for each gene and measured in triplicate. Three no-template controls were run for each quantitative PCR assay, and genomic DNA contamination of total RNA was controlled using RT minus controls (samples without the reverse transcriptase).

Semiquantitative PCR

Conventional PCR amplifications were conducted in a 25 μL solution containing 1 × PCR buffer, 0.2 mM dNTP mix (Invitrogen, Carlsbad, CA, USA), 1.5 mM magnesium chloride, 400 nM of each primer and 1 U of DNA polymerase and 2 μL of cDNA template, corresponding to 5 μg total RNA in a 20 μL final volume. Negative control of amplification was performed with 2 μL of water instead of cDNA template. Amplification conditions were: 2 min at 95°C, 11 cycles of 30 s at 95°C, 30 s at 61°C, decreasing 0.5°C every cycle, and 20 s at 72°C, followed by 23 cycles of 30 s at 95°C, 30 s at 55.5°C, and 20 s at 72°C, and a final extension of 2 min at 72°C. Reactions were carried out in a thermal cycler. 10 μL of the PCR products were resuspended in 6X loading buffer [30% glycerol, 0.5 μg/mL ethidium bromide (BrEt)] and electrophoresed through 1.5% agarose in 0.5X Tris/Borate/EDTA (TBE) buffer (45 mM Tris-borate; 1 mM Na_2EDTA pH 8.0) with 0.5 μg/mL ethidium bromide (BrEt) for 1.5 h.

Statistics

Data were expressed as mean ± SEM values of three or four independent experiments with different cell batches, each one performed in duplicate or triplicate. Statistical comparisons were assessed by using one-way ANOVA (Scheffe's *F*-test) followed in some instance by a two-way ANOVA test. Differences were accepted as significant as $p < 0.05$ or less.

Results

Chromaffin cells express nNOS and iNOS both showing different regulation by cytokines and glucocorticoids

In order to study the possible participation of different NOS isoforms in chromaffin cell death-induced by IFNγ, we first investigate the expression of specific NOS isoforms in these cells, at both protein and mRNA levels. As shown in Fig. 1 western blot experiments for chromaffin cells treated with cytokines or dexamethasone for 24 h indicate the expression of two NOS isoforms, nNOS and iNOS, nNOS being basally expressed. Both NOS isoforms showed a different regulation by cytokines and dexamethasone. Thus, while nNOS expression was not significantly regulated by cytokines, it was activated by dexamethasone in a concentration-dependent manner (Fig. 1b). iNOS was induced by cytokines, especially by IFNγ, but not by dexamethasone (Fig. 1a).

In order to go deeper in the study of NOS isoform expression and its specific regulation, PCR [semiquantitative for Fig. 2(a) and quantitative for Fig. 2(b and c)] experiments were set for the nNOS, iNOS, eNOS, and G3PDH genes. Semiquantitative RT-PCR experiments confirm the presence of specific mRNAs for nNOS and iNOS (Fig. 2a). Data also showed a small, mild presence of eNOS mRNA, this probably due to a small contamination in the chromaffin cell cultures with endothelial cells, although as counted at the moment of platting, this contamination in never higher as 5%. So, our data indicate that the main isoforms expressed

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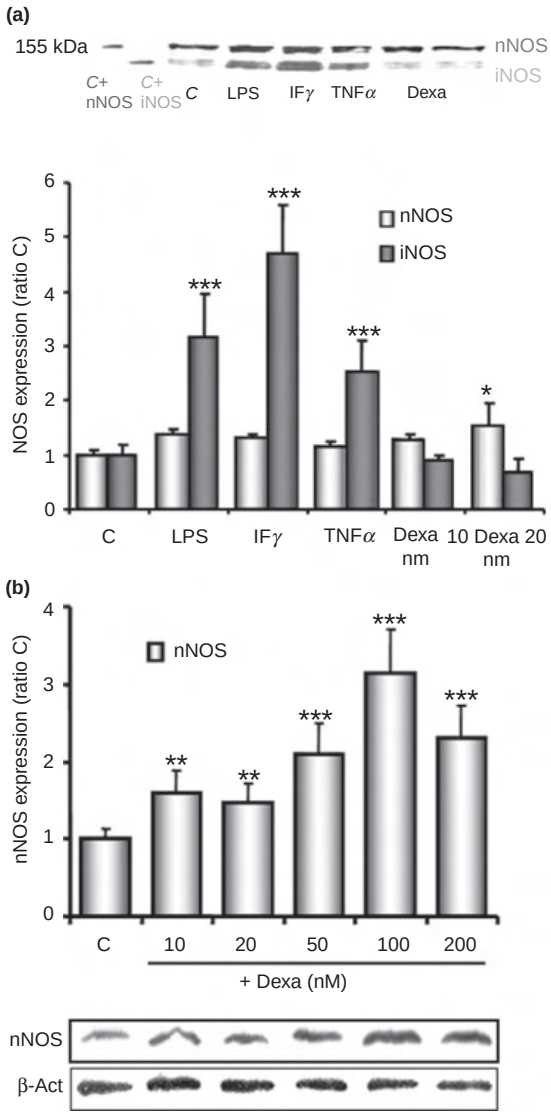

Fig. 1 Regulation of nNOS and iNOS expression by LPS, IFNγ, TNFα, and glucocorticoids at protein level. Bovine chromaffin cells were incubated for 24 h in the absence (control) or presence of 10 μM LPS, 10 nM IFNγ, or 10 nM TNFα (Prepotech, London, UK) (a) or indicated concentrations of dexamethasone (b). NOS expression levels were measured by western blot with antibodies anti-nNOS and anti-iNOS (BD-Biosciences, San Diego, CA, USA), both with positive controls. Data are expressed as a quantification of results over control and are mean ± SEM values of three experiments. Statistic compares the effect of cytokines (a) or dexamethasone (b) with respective control values (c). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) (one-way ANOVA test).

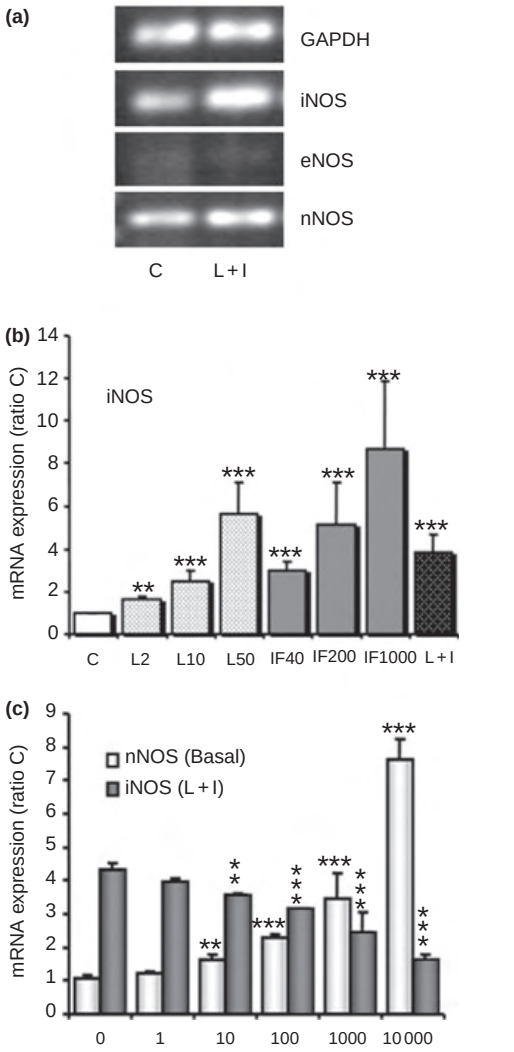

Fig. 2 mRNA expression and regulation of different NOS isoforms in bovine chromaffin cells. (a) For conventional RT-PCR bovine chromaffin cells were incubated for 6 h in the presence or absence of LPS 10 μM plus IFNγ 10 nM (L + I). mRNA was isolated and treated with appropriate set of primers for nNOS, eNOS, iNOS, and GAPDH as a control, as described in Materials and Methods. (b and c) For real time RT-PCR bovine chromaffin cells were incubated for 6 h with (b) Increasing concentrations of LPS (2–50 μg/mL; 2–50 μM) or IFNγ (40–1000 ng/mL; 2–50 nM) or (c) Increasing concentrations of dexamethasone (1 nM–10 μM) in the absence (nNOS) or presence of LPS 10 μg/mL plus IFNγ 200 ng/mL (iNOS) and mRNA was extracted and treated with appropriate sets of primers for nNOS and iNOS, and GAPDH as a control, in an AB 7900 HT Fast Real PCR from Applied Biosystems, as described in Materials and Methods. Data are expressed as ratios over their respective controls and are mean ± SEM values obtained from three experiments each one performed in triplicate. Statistic compares the effect of cytokines (b) or dexamethasone (c) with their specific controls (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) (one-way ANOVA test).

on primary cultures of bovine chromaffin cells are nNOS and iNOS.

In Fig. 2(b and c), we present data on mRNA NOS expression obtained by quantitative real-time PCR tech-

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niques after chromaffin cell incubation with increasing concentrations of lipopolysaccharide (LPS) or IFNγ (Fig. 2b) or increasing concentrations of dexamethasone, in the absence (nNOS) or presence of LPS plus IFNγ (iNOS) (Fig. 2c). These data show that both LPS and IFNγ increased mRNA iNOS expression in a dose-dependent manner (Fig. 2b) while these treatments do not significantly affect nNOS mRNA expression (data not shown). Moreover, treatment with dexamethasone increased mRNA nNOS basal expression but decreased mRNA iNOS in a concentration-dependent manner (Fig. 2c). So, these results confirm the expression of specific nNOS and iNOS isoforms in chromaffin cells with different regulation by cytokines and glucocorticoids at both protein and mRNA levels.

Chromaffin cells express a calcium-dependent basal NOS activity and a calcium-independent IFNγ-induced NOS activity, which could be specifically inhibited by different NOS inhibitors

As it is known, constitutively-expressed nNOS activity is calcium dependent and iNOS activity-induced by cytokines is mainly calcium independent. In order to confirm the presence of both, calcium-dependent and independent NOS activity in chromaffin cells, NOS activity was measured by the quantification of the production of [U-^{14}C]L-citrulline from [U-^{14}C]L-arginine, as described (Vicente *et al.* 2002), both in the presence of physiological extracellular calcium and in the presence of EGTA plus calmidazolium (1-[bis (4-chlorophenyl)methyl]-3-[2-(2;4-dichlorophenyl)-2-{2;4-dichlorobenzyloxy}ethyl]-1H-imidazolium chloride), a CaM blocker, in the absence or presence of IFNγ. As shown in Fig. S1A, in the presence of 2..5 mM extracellular calcium, total basal NOS activity was 0.63 ± 0.04 nmol [^{14}C]citrulline/10^6 cells in 24 h. This activity was reduced by 70% in the absence of calcium. The stimulation of chromaffin cells with 10 nM IFNγ increases by 40% the total NOS activity, this effect being stronger (about 75%) in the absence of calcium. By contrast, calcium-dependent NOS activity was not significantly affected by IFNγ (Fig. S1A). To evaluate the specificity and relative potency of different NOS inhibitors on both Ca^{2+}-dependent (nNOS) and Ca^{2+}-independent (iNOS) activities, we measured NOS activity in the presence of different concentrations. For this study *N*-(3-aminomethyl)benzyl) acetamidine (1400W) as a specific iNOS inhibitor (Garvey *et al.* 1997), *N*-ω-propyl-L-arginine (*N*-PLA) a very high specific nNOS inhibitor (Zhang *et al.* 1997; Fedorov *et al.* 2003) and *S*-methyl-L-thiocitrulline, *N*-methyl L-arginine (L-NMA) and 3-Bromo-7-nitroindazol (7-NI) as less specific nNOS inhibitors (Vicente *et al.* 2002) were used. These compounds produced a dose-dependent inhibition of NOS activity with IC50s for different inhibitors indicated in Fig. S1B. The order of potency of inhibitors on total NOS activity was: *N*-PLA > W-(1400) > thiocitrulline = 7-NI > L-NMA. Similar results were obtained by measurement of nitrites in extracellular medium (data not shown). In order to demonstrate inhibition specificity on calcium-dependent (nNOS) or calcium-independent (iNOS) activity of different inhibitors, NOS activity was measured in the presence or absence of calcium for three of these inhibitors: 1400W, *N*-PLA and thiocitrulline. Results obtained with these inhibitors and IC50 values are shown in Fig. S1C–E. These results clearly demonstrated that, in this cell type, W-1400 was a more specific inhibitor of Ca^{2+}-independent NOS activity (IC50 = 0.27 ± 0.09 μM) than Ca^{2+}-dependent one (IC50 = 11.5 ± 2.9 μM), while *N*-PLA and thiocitrulline were better inhibitors of Ca^{2+}-dependent activity (IC50 = 0.098 ± 0.007 and 12.3 ± 3.6 μM, for *N*-PLA and thiocitrulline respectively) than of the Ca^{2+}-independent activity (178.9 ± 56.8 and 157.8 ± 53.6 μM). Therefore, W-1400 (0.1-10 μM) could be used in our system as a specific inhibitor of iNOS and *N*-PLA (0.1-10 μM) and thiocitrulline (1-100 μM) as specific nNOS inhibitors.

IFNγ-induced chromaffin cell death is totally reverted by iNOS inhibitors but partially by nNOS inhibitors

As results above indicate the expression of two isoforms, nNOS and iNOS, in chromaffin cells, the specific participation of nNOS and iNOS on cell viability and apoptosis-induced by cytokines was investigated by challenging the bovine chromaffin cells with IFNγ in the absence or presence of different NOS inhibitors. 10 nM IFNγ (200 ng/mL) decreased cell viability in about a 65% (Fig. 3a). This effect was reverted, in a dose-dependent manner, by NOS inhibitors. Concentrations in the range of 1–5 μM of W-1400 were able to totally revert IFNγ effects, whereas higher concentrations (up to 1000 μM) were needed in the case of nNOS-specific inhibitors.

The increase in NO production induced by IFNγ (1.52 ± 0.11 times the NO basal levels), was also inhibited by NOS inhibitors but in a manner not always parallel to their ability to decrease cell viability. Thus, W-1400 restored nitrite production and cell viability to their basal levels, even at very small concentrations (Fig. 3a and b). *N*-PLA drastically reverted NO production bellow the basal levels but had a mild effect on cell viability at small concentrations; similar effects were observed with thiocitrulline and 7-NI (Fig. 3c and d). Thus, while 1400 W, a specific iNOS inhibitor, was able to inhibit completely both cell death and increase in NO levels-induced by IFNγ at 1–10 μM concentration, nNOS inhibitors needed very high concentrations (up to 1000 μM) to get the same or lower effects. At a concentration of 10 μM, the strength of these inhibitors was as follows: W-1400 > thiocitrulline = L-PLA > 7-NI > L-NMA. These results point out that the diminution in cell viability-induced by IFNγ was mainly due to iNOS activation.

In order to know whether IFNγ-induced increase in cell death is due to apoptosis, and the implication of NOS isoforms, we tested the action of these NOS inhibitors on

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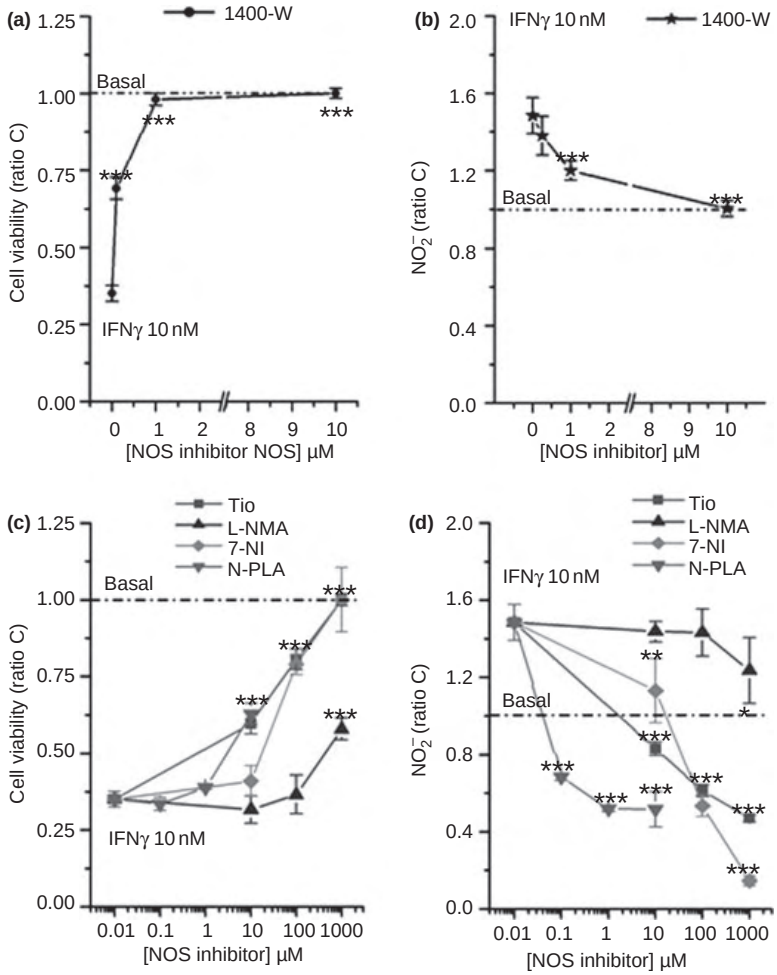

Fig. 3 Effect of different concentrations of NOS inhibitors on IFNγ-induced increases in NO levels and decreases in cell viability. Bovine chromaffin cells were incubated for 24 h with the indicated concentrations of NOS inhibitors (W-1400, thiocitrulline, L-NMA, 7-NI, *N*-PLA) in the presence of 10 nM (200 ng/mL) IFNγ. NO levels (a and c) were measured by a 2,3-diaminonaphtalene spectrofluorimetric assay and cell viability (b and d) by the XTT spectrofluorimetric assay as described in Materials and Methods. Data were expressed as ratios over control and are mean ± SEM values obtained from three experiments each one performed in quadruplicate. [NO]basal = 0.72 ± 0.07 nmol/10^6 cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (one-way ANOVA test).

apoptosis induced by IFNγ. Data from Fig. 4 show that apoptosis-induced by 10 nM IFNγ (about three times the basal values) was completely inhibited by 1400W 10 μM, while *N*-PLA, thiocitrulline and L-NMA, even at doses 100 times higher, were not able to induce a total reversion of apoptosis. From these results, it seems fair to conclude that the main NOS isoform involved on IFNγ-induced apoptosis is iNOS, with minor effect of nNOS.

Cytokines activate the translocation to the nucleus of NFκB which mediates activation of iNOS expression

It is well known in the literature that cytokines activate iNOS expression by the early activation of some nuclear transcriptional factors, including NFκB (Aktan 2004). Moreover, it is also known that physiological levels of NO, similar to those produced by the basal activity of nNOS or eNOS, prevent induction of iNOS mRNA expression through the suppression of NFκB activation (Colasanti *et al.* 1995; Togashi *et al.* 1997). Therefore, in order to study in depth the possible mechanism by which each NOS isoform could contribute to NO effect on cell viability and apoptosis of chromaffin cells, we study the implication of NFκB on IFNγ-induced apoptosis and on iNOS activation. Because it is known that NFκB translocation to nucleus and activation require phosphorylation and degradation of its specific inhibitor nuclear factor κB inhibitor (IκB), which complexed NFκB into the

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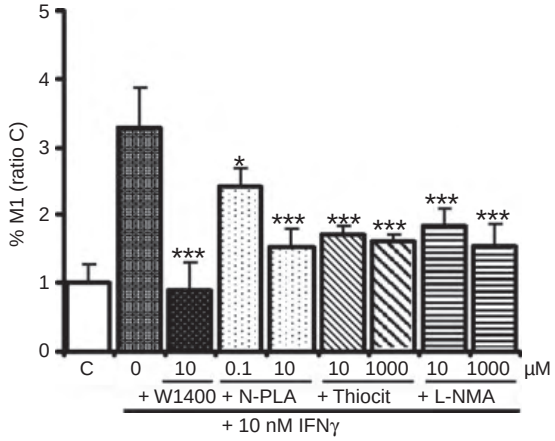

Fig. 4 Effect of NOS inhibitors on IFNγ-induced-apoptosis in chromaffin cells. Bovine chromaffin cells (2×10^6) were incubated for 24 h with the indicated concentrations of NOS inhibitors in the presence of 10 nM IFNγ. Apoptosis was measured by flow cytometry as described in Materials and Methods. Data were expressed as ratios over basal (3.4 ± 0.40% M1) and are mean ± SEM values obtained from three experiments each one performed in duplicate. Statistic compares the effect of NOS inhibitors in the presence of IFNγ with the apoptosis induced by IFNγ alone (*$p < 0.05$; ***$p < 0.001$) (one-way ANOVA test).

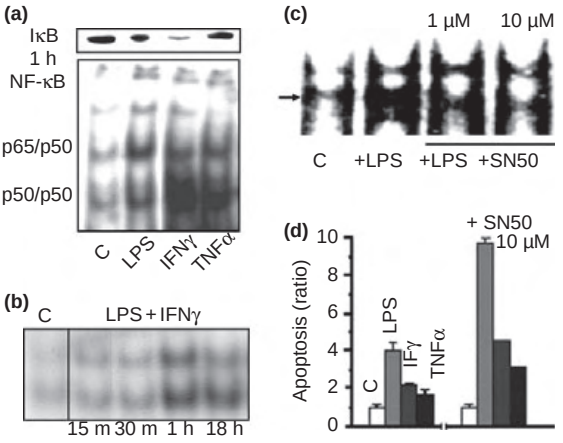

Fig. 5 Effect of cytokines and SN50 on NFκB translocation to the nucleus and apoptosis. Bovine chromaffin cells were incubated for 1 h with different cytokines (LPS 10 μM, IFNγ 10 nM, TNFα 10 nM) (a) or with LPS + IFNγ at indicated times (b) and IκB degradation in cytosol measured by western-blot ('a' up) or NFκB binding to DNA from nuclear extracts isolated and electrophoresed in EMSA ('a' down and b) was measured as described in Materials and Methods. The effect of SN50 (Calbiochem, San Diego, CA, USA) (1 and 10 μM) on NFκB activity induced by LPS (c) or apoptosis-induced by cytokines (d) was determined. Apoptosis data were expressed as ratios over control. Effects of SN50, in (c) and (d), were statistically significant against both, basal and respective cytokines without SN50, at $p > 0.001$ (multi-variance analysis of ANOVA test). (a–c) show a representative experiment of three. In (d) data are mean ± SEM values of three experiments performed by duplicate.

cytosol, we performed both electrophoretic mobility shift assays (EMSA) and western blot experiments to check out NFκB translocation to the nucleus and IκB degradation on the cytosol, respectively. Results from Fig. 5(a) show that LPS, IFNγ and TNFα were able to increase IκB degradation, as observed on the western analysis on the top of figure, and also to increase NFκB translocation to the nucleus where it binds to nuclear DNA (Fig. 5a and b).

To check out whether cytokine-induced NFκB activation was specific, we studied this in the presence and absence of the membrane permeable peptide SN50 (1 and 10 μM), which contains the nuclear localization signal (NLS) of NFκB and blocks the intracellular recognition mechanism for the nuclear localization signal (NLS) on NFκB dimmers thereby preventing their nuclear translocation (Lin *et al.* 1995). Results in Fig. 5(c) show that SN50 was able to inhibit NFκB activation in a dose-dependent manner. However, apoptotic effect of LPS and cytokines was enhanced and not inhibited by SN50, that indicating that NFκB could be activating survival genes in addition to death genes, but that NFκB activation is not enough to stop chromaffin cells apoptosis induced by cytokines. In order to know if NFκB activation could be mediate iNOS expression induced by IFNγ we study the SN50 effect on the increase in iNOS mRNA and protein expression induced by LPS + IFNγ. Results from Fig. 6 indicate that SN50 was able to inhibit iNOS expression at transcriptional level in a dose-dependent manner, with 30% inhibitory effect at 10 μM concentrations (Fig. 6a). The same inhibitory effect (about 50%) was shown at level of protein expression (Fig. 6b). These data are in agreement with those from enzymatic activity where 10 μM SN50 was able to reduce calcium-independent NOS activity induced by LPS + IFNγ by 50% ($C = 0.53 \pm 0.07$ nmol [U-^{14}C]L-citrulline/10^6 cells; + 10 μM SN50 = 0.36 ± 0.04 nmol/10^6 cells).

nNOS inhibitors shorten the time of the IFNγ-induced translocation to the nucleus of NFκB and increase by themselves NFκB activation

Because our results above suggested some involvement of nNOS in IFNγ effects on chromaffin cell death and apoptosis, in order to know the possible participation of nNOS in the IFNγ effects on NFκB nuclear translocation we studied the effect of different NOS inhibitors on NFκB translocation to the nucleus, in basal conditions and in the presence of LPS + IFNγ. Chromaffin cells were challenged, at the indicated times, with LPS plus IFNγ in the presence and absence of nNOS (thiocitrulline, *N*-PLA) and iNOS (W-1400) inhibitors. As seen on Fig. 7(a), LPS + IFNγ induced NFκB translocation to the nucleus (2–3 times basal levels) at long times, 1–18 h. Addition of W-1400 had not a significant effect on NFκB activation at these times. However, the

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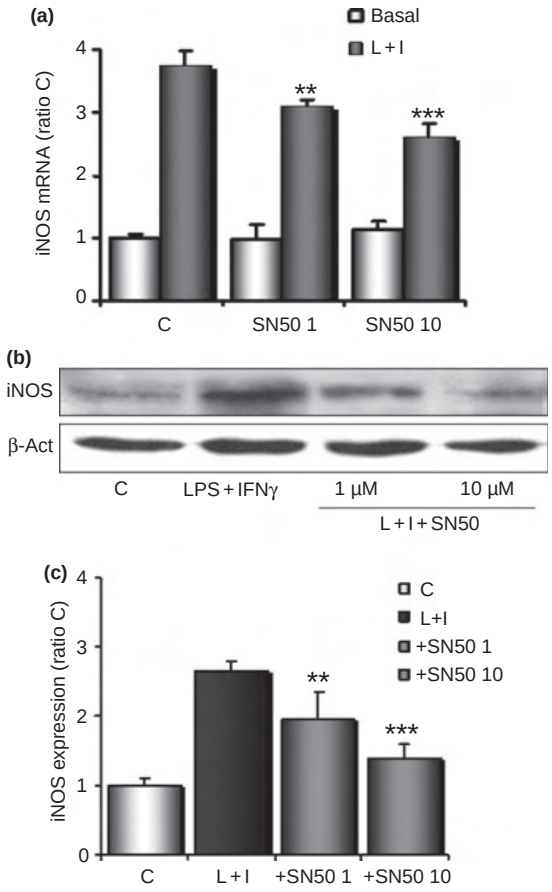

Fig. 6 Effect of SN50 on iNOS expression at both mRNA (a) and protein (b and c) level. Bovine chromaffin cells were incubated for 24 h with LPS 10 μM plus IFNγ 10 nM in the absence or presence of 1–10 μM SN50 and mRNA or proteins was extracted as indicated in Materials and Methods. Data in (a) and (c) are expressed as ratios over basal control and are mean ± SEM values of two experiments each one performed in triplicate. In (b) a representative western blot is shown. Statistic compares the effect of SN50 on iNOS mRNA or iNOS protein induced by LPS + IFNγ (**$p < 0.01$; ***$p < 0.001$) (one-way ANOVA test).

presence of the specific nNOS inhibitors (*N*-PLA and Thiocitrulline) shortened the time of translocation of NFkB to the nucleus to 15–30 min (Fig 7a).

On the other hand, in the absence of IFNγ stimuli, nNOS inhibitors basally induced NFkB activation (Fig. 7b). Moreover, these inhibitors were able to induce, by themselves, a slight increase of 30% in iNOS gene expression (data not shown).

IFNγ induces ^{847}Ser nNOS phosphorylation and ^{727}Ser STAT-3 phosphorylation and translocation to the nucleus

One of the more probable mechanisms used by IFNγ to inactivate nNOS generating low NO levels, stimulate NFκB activation and thus up-regulate the iNOS mRNA expression, is the phosphorylation of nNOS. In order to know if this mechanism is mediated by IFNγ in chromaffin cells, we studied nNOS phosphorylation on serine 847 at different times and with several cytokines. Western blot results shown on Fig. 8(a) indicate that LPS, IFNγ and TNFα, and the combination LPS + IFNγ were able to increase nNOS phosphorylation in ^{847}Ser while they do not have any significant effect or even decrease nNOS expression. nNOS phosphorylation was time-dependent, maximal effect being achieved after 1 h stimulation for all the stimuli evaluated (Fig. 8b and c, left panel). These results are consistent with results obtained on NFκB activation, shown above, and with maximal effect of NOS inhibitors on nNOS activity (Vicente *et al.* 2002). Thus, taken all together, these data suggest that IFNγ activates iNOS expression, inducing NFκB translocation to the nucleus and these effects might be mediated by nNOS phosphorylation.

Because one of the more important signal transduction pathways by which IFNγ induces its apoptotic and inflammatory effects, as well as NFκB activation, is the canonical JAK/STAT pathway, largely responsible for the antiviral and growth-inhibitory activities of interferons and iNOS activation (Stempelj *et al.* 2007), we study the possibility that IFNγ was able to activate STAT-3 phosphorylation. Results in Fig. 8(b and c, middle panel) shown that IFNγ was able to activate STAT-3 phosphorylation in ^{727}Ser, maximal effect being at 1 h stimuli, time at which p-STAT3 translocation to the nucleus was maximal too (Fig. 8b and c, right panel).

JAK/STAT pathway is involved in both nNOS phosphorylation and activation of iNOS expression induced by IFNγ

In order to know if JAK/STAT3 pathway activation induced by IFNγ could be mediated by IFNγ induction of nNOS phosphorylation and if activation of this pathway could mediate the increase in activation of iNOS expression induced by IFNγ, we tested the effect of specific inhibitors of JAK/STAT pathway on both, nNOS phosphorylation and iNOS mRNA expression induced by LPS + IFNγ, these effects being compared to the effects of another protein kinase inhibitors. Results from Fig. 9(a) show that nNOS phosphorylation induced by LPS + IFNγ was highly inhibited by the specific JAK family inhibitor JAKI1 (100 nM) and by AG490 (2 μM), a JAKs family tyrosine kinase inhibitor which inhibit STAT-3 activation mainly by JAK2, as well as by 5 μM PD98059, 1 μM KT5823 and 10 μM H89, concentrations at which these compounds specifically inhibit Mitogen Activated Protein Kinase Kinase (MEK or MAPKK), cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA) activity, respectively, but not by 5 μM LY294002, a specific inhibitor of PI3K (Fig. 9a). These data suggest the participation of JAK pathway, as well as some other Ser-Tre kinases, in the

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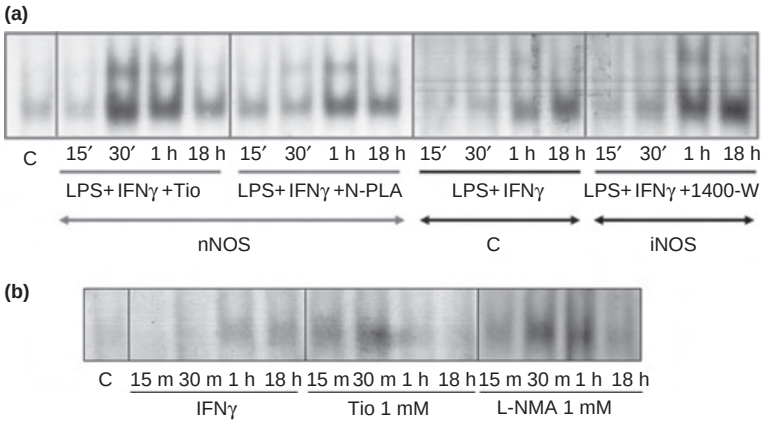

Fig. 7 Effect of NOS inhibitors on LPS plus IFNγ-induced (a) or basal (b) NFκB translocation to the nucleus in bovine chromaffin cells. Bovine chromaffin cells (5×10^6 cells/condition) were challenged for indicated times with LPS 10 μM + IFNγ 10 nM, in the absence or presence of the specific NOS inhibitors W-1400 0.1 μM, thiocitrulline 10 μM or *N*-PLA 10 μM (a) or with indicated NOS inhibitors at 1 mM concentrations (b). Nuclear extracts were isolated and electrophoresed in an EMSA, as described in Materials and Methods. A representative experiment of three is shown.

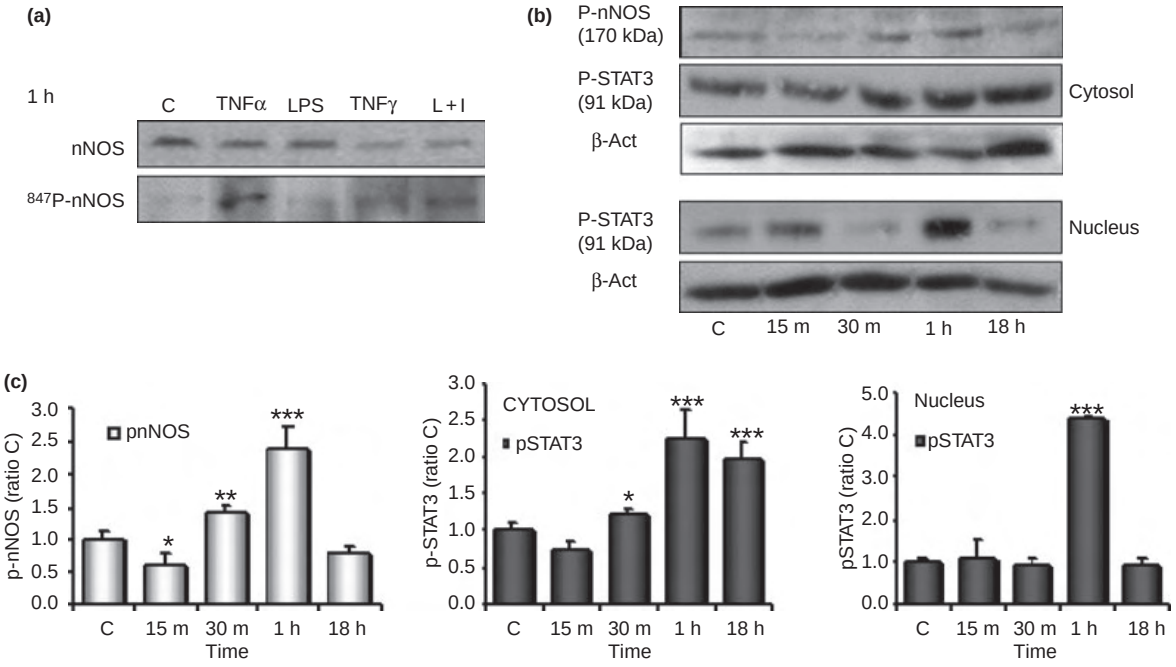

Fig. 8 Cytokine induction of 847Ser nNOS phosphorylation and 727STAT-3 phosphorylation in bovine chromaffin cells. Bovine chromaffin cells were challenged for 1 h with LPS 10 μM, IFNγ 10 nM, TNFα 10 nM or a combination of LPS plus IFNγ (a) or with IFNγ at indicated times (b) and proteins from cytosolic P-nNOS (a and b) or cytosolic P-STAT-3 and nuclear P-STAT-3 (b) was measured by western blot techniques using specific antibodies [Abcam (Cambridge, UK) and Santa Cruz Biotechnology, Inc. (Heidelberg, Germany), respectively]. A representative experiment of three is shown. (c) Quantification of results for three different experiments. Statistic compares results obtained at different times over basal control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) (one-way ANOVA test).

activation of nNOS phosphorylation induced by IFNγ in chromaffin cells. However, only the inhibition of the STAT3 signaling pathway by 2 μM AG490 or 1 μM JAKI1, was able to inhibit by 90% and 50%, respectively, the mRNA iNOS expression, without affecting nNOS expression, while the above indicated PKA, PKG, and MEK inhibitors did not have a significant inhibitory effect on mRNA iNOS expression (Fig. 9b).

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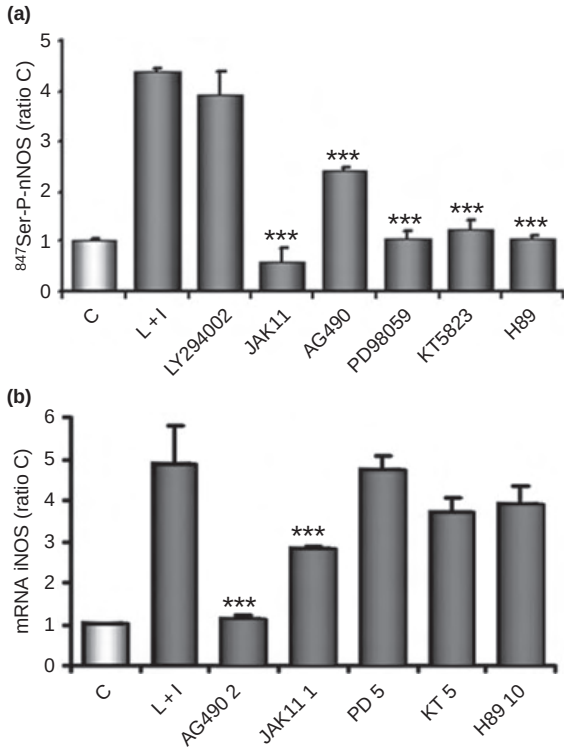

Fig. 9 Effect of different protein kinase inhibitors on nNOS phosphorylation at ^{847}Ser and on iNOS mRNA expression. Bovine chromaffin cells were challenged with a combination of LPS 10 μM plus IFNγ 10 nM in the absence or presence of protein kinase inhibitors (at concentrations indicated in the text) and ^{847}Ser p-nNOS levels (1 h) (a) and mRNA iNOS expression (6 h) (b) were measured by western blot o real time PCR techniques as is described in Materials and Methods. Data were expressed as a quantification of results over control without LPS + IFNγ. Statistic compares the effect of protein kinase inhibitors on nNOS phosphorylation (a) or mRNA iNOS expression (b) induced LPS plus IFNγ alone. (***$p < 0.001$) (one-way ANOVA test).

Discussion

Recent studies carried out by our group postulated that both, exogenous NO and endogenous NO, triggered by cytokines, induce apoptosis in chromaffin cells (Vicente *et al.* 2006; Pérez-Rodríguez *et al.* 2007). NO donors, therefore exogenous NO, induce apoptotic cell death mediated by NO and peroxynitrites, conclusions also supported in the literature (Nomura 2004). However, the effect of endogenous NO, which in these cells can be generated by cytokines (Turquier *et al.* 2002; Vicente *et al.* 2006) or glutamate (González *et al.* 1998; Arce *et al.* 2004), on apoptosis is more controversial, as NO can be synthesized in most cell types via different NOS isoforms (nNOS, eNOS, and iNOS), having these isoforms a tissue-specific localization and different effects on cell death and survival. Neurons mainly express nNOS (Förstermann *et al.* 1998), but also iNOS in pathological conditions such as trauma, inflammation and ischemia (Bredt 1999). In this work, we study the effect of endogenously NO generated by IFNγ on cell viability and apoptosis of chromaffin cells and study the possible involvement of both, nNOS and iNOS isoforms, in chromaffin cell death.

Chromaffin cells express both nNOS and iNOS

Our studies on the expression of specific NOS isoforms in chromaffin cells at both transcriptional and translational levels permit us to assess here the presence in bovine chromaffin cells of at least two NOS isoforms, nNOS and iNOS, having a different regulation. We outlined that iNOS expression, at protein level, is induced by cytokines while nNOS is not. The use of more accurate techniques in further essays, such as real-time PCR, permits us to define the regulation of both isoforms better. Thus, IFNγ or LPS, alone or in combination, induce a dose-dependent increase in the mRNA level of iNOS, effect that is abolished by dexamethasone in a dose–response manner, as described in many other cell types (Korhonen *et al.* 2002; Golde *et al.* 2003; Shinoda *et al.* 2003). On the other hand, nNOS expression is up-regulated by dexamethasone at both protein and mRNA levels. It has been described that dexamethasone inhibit the expression of inflammatory genes like iNOS, by a mechanism such as destabilizing iNOS mRNA (Korhonen *et al.* 2002), post-transcriptional level (Shinoda *et al.* 2003) or reduction in protein synthesis (Golde *et al.* 2003). However, the effects of dexamethasone on nNOS expression are more controversial. So, although there are many data on the literature indicating a decrease by glucocorticoids on nNOS expression in neuroblastoma cell lines (Schwarz *et al.* 1998) and in endotoxemic neonate rat brain (Wang *et al.* 2005) or rat lung, liver, and aorta of the rat (Knowles *et al.* 1990), there are also data in the literature showing an activation of nNOS expression by dexamethasone or an increase in its activity induced by glucocorticoids in cerebellar glial cells (Baltrons *et al.* 1995). Thus, it seems that nNOS expression is subjected to differential tissue-specific mechanisms. In this way, as the presence of glucocorticoid-responsive elements (GRE) in nNOS promoter has not been demonstrated, it is possible that the dexamethasone action on nNOS gene expressed in chromaffin cells is not a direct action but the result of interaction with other transcriptional factors like cAMP-response element binding protein (Zhong and Minneman 1993). On the other hand, the effect of glucocorticoids increasing nNOS expression and activity could produce very high NO levels which could repress NFκB activation and inhibit iNOS expression. These results suggest us a model where basal NO produced by nNOS could inhibit NFκB translocation to the nucleus, regulating iNOS expression.

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iNOS is the NOS isoform mainly involved in chromaffin cell death

Our results here show that in chromaffin cells both, calcium dependent basal NOS activity (nNOS) and calcium-independent NOS activity-induced by IFNγ (iNOS), are found. In these cells W-1400, at doses between 1 and 10 μM, is a specific inhibitor of only iNOS and *N*-PLA (0.1-100 μM) or thiocitrulline (10–100 μM) are specific only of nNOS. Therefore, we used these inhibitor concentrations to study the participation of NO generated by nNOS and iNOS in chromaffin cell viability and apoptosis.

On the basis of results showing that iNOS inhibitor W-1400 completely reverted IFNγ-induced increase in NO levels and decrease in cell viability but nNOS inhibitors only partially reverted these effects, we conclude that iNOS is the main NOS isoform involved in cytokine-induced chromaffin cell death with minor participation of nNOS. The same results were obtained on apoptosis, that is, iNOS inhibitors were the strongest inhibitors of apoptosis induced by IFNγ.

NFκB activation and nNOS phosphorylation are involved in the mechanism of iNOS induction by IFNγ

The implication of transcriptional factor NFκB on iNOS regulation is well documented (Aktan 2004). We observed that cytokines, activators of apoptosis, do activate NFκB, promoting its translocation to the nucleus. However, the fact that an increase of apoptosis-induced by cytokines is produced when NFκB translocation to the nucleus is avoided, indicates that NFκB might be a survival factor, probably because it activates other survival gene in addition to iNOS activation.

In respect to NFκB participation on iNOS mRNA and protein expression, our studies with SN50, a specific inhibitor of NFκB translocation into the nucleus, indicate that SN50 was able to inhibit iNOS expression at both transcriptional and translational level, but only about 30–50% at SN50 doses used. These results suggest that NFκB participation in iNOS induction is not the only mechanism mediating iNOS gene expression and indicate that this transcriptional factor could also be mediating the expression of other survival genes which counteract death genes mediating apoptosis.

Regarding the possible participation of nNOS in IFNγ induction of iNOS and apoptosis through NFκB, some authors defend an interesting perspective of apoptotic regulation, that is, cytokine induction of iNOS is regulated by nNOS through NFκB (Colasanti *et al.* 1995; Togashi *et al.* 1997). Our results show that different nNOS inhibitors induced NFκB activation, showing that nNOS inhibition in basal conditions was enough to activate NFκB and thus iNOS expression. Therefore, basal NO levels, generated by constitutively expressed nNOS, could block NFκB activation and thus, iNOS gene expression. Taken these data all together with the increase in NFκB translocation to the nucleus induced by IFNγ, we can deduce that IFNγ activates iNOS expression, inducing NFκB translocation to the nucleus, this effect being regulated by nNOS.

Nitric oxide has been shown to affect the activity of NFκB and other transcriptional factors through *S*-nitrosylation. In the case of NFκB, NO mainly affects their transcriptional activities indirectly, by *S*-nitrosylation of IκB kinases (IKKs), that preventing the phosphorylation and degradation of IκB and thus, inhibiting the activation of NFκB pathway (Reynaert *et al.* 2004; Kenny and Chung 2006). In our model, NO donors inhibited NFkB activation and iNOS expression (data not shown). So, the low NO levels generated by cytokines or by NOS inhibitors could increase NFkB activation probably by avoiding NO-induced *S*-nitrosylation of NFkB.

Our results agree with a very interesting hypothesis brought up in the literature. This model proposes that iNOS expression is regulated by nNOS in the following manner: constitutive NO produced by nNOS would be enough to avoid NFκB translocation to the nucleus. However, once there, NFκB would activate iNOS expression, producing enormous amounts of NO and apoptosis. In this model, cytokines would inhibit nNOS via phosphorylation, avoiding NO formation and favoring NFκB translocation to the nucleus (Mariotto *et al.* 2004; Conti *et al.* 2007). To test the accuracy of this hypothesis on our cell model, chromaffin cells were subjected to cytokine challenge and nNOS phosphorylation was measured. nNOS isoforms have got different sites for serine or tyrosine phosphorylation. nNOS can be phosphorylated by PKA, PKG and Ca^{2+}/calmodulin-dependent protein kinase II (Bredt *et al.* 1992). Phosphorylation of nNOS by both PKG and PKA diminishes its catalytic activity (Dinerman *et al.* 1994). Down-regulation of the nNOS activity by phosphorylation leads to a lower NO concentration, and therefore to NFκB activation and iNOS expression. Phosphorylation of nNOS in serine 847 has been widely studied, providing a down-regulation of activity in many cell types (Nakane *et al.* 1991; Hayashi *et al.* 1999). Here, we observed that cytokine challenge of chromaffin cells provided an increase in the nNOS phosphorylation in ser847, maximal at 1 h. These results occur in parallel to maximal NFκB translocation into the nucleus after the same stimuli, pointing at a possible interaction between both events. The serine threonine kinases PKA, PKG could be involved in nNOS phosphorylation as specific inhibitors of these enzymes were able to strongly inhibit this cytokine effect. Also, the dual kinase MEK, but not PI3K, could be involved in this Ser-nNOS phosphorylation, probably via extracellular signal-regulated kinases (ERKs) because specific inhibitors of p38MAPKs and c-Jun N-terminal kinases (JNKs) were much less effective on Ser-nNOS phosphorylation inhibition than MEK inhibitor PD98059 (data not shown).

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JAK/STAT pathway is involved in the mechanism of iNOS induction by IFNγ

Another pathway involved in iNOS expression has been described for the JAK/STATs family (Bolli *et al.* (2003) and Stempelj *et al.* (2007). The human NOS II promoter contains consensus sequences for the binding of transcription factors, including IFNγ regulatory factor-1, STAT binding to interferon-gamma activated sites (GAS) elements, activator protein 1 (AP-1), NFκB, and others (Spitsin *et al.* 1996; Linn *et al.* 1997). In murine messagial cells it has been described that there might be a cross-talk between the STATs and NFκB transcription factors. Thus, STAT3, via direct interactions with NF-kappaB p65, serves as a dominant-negative inhibitor of NF-kappaB activity to suppress indirectly cytokine induction of the iNOS promoter in these cells (Yu *et al.* 2002). Although current knowledge on the role of STAT3 concerning regulation of the human iNOS gene is still fragmentary, the recent use of interfering RNA technology identified STAT3 as being crucial for up-regulation of iNOS, demonstrating that the interleukin-22/STAT3 pathway potentiates expression of iNOS in human colon carcinoma cells (Ziesché *et al.* 2007). As observed with nNOS phosphorylation, our experiments report an increase in STAT3-phosphorylation and p-STAT3 nuclear translocation maximal at 1 h after IFNγ stimulation of chromaffin cells. Moreover, JAKI1, a specific inhibitor of JAK family tyrosine kinases, and AG490, a selective inhibitor of STAT-3 phosphorylation by JAKs, were able to inhibit both nNOS phosphorylation and iNOS mRNA expression induced by IFNγ, while other protein kinase inhibitors were only able to inhibit nNOS phosphorylation without affecting iNOS mRNA expression.

So, on the basis of these results, we point at a possible p-nNOS-p-STAT3-NFκB cross-talk in chromaffin cells, JAK/STAT pathway potentiating iNOS expression probably by increasing nNOS phosphorylation and NFκB activity.

In short, at the view of results from this paper, we put forward that endogenous NO has a very important role on the regulation of apoptosis of chromaffin cells and we propose a model to explain the effects of endogenous NO, generated by IFNγ stimulation, on cell death of bovine chromaffin cells.

- In basal conditions, chromaffin cells express only nNOS. Physiological concentrations of NO produced by nNOS would be enough to stop NFκB activation, as it has been observed that nNOS inhibitors promote its activation.
- IFNγ, probably by promoting tyrosine kinase activity of JAK, induces nNOS phosphorylation on bovine chromaffin cells, diminishing NO basal levels, thus allowing NFκB translocation to the nucleus, and DNA binding. This would, in turn, activate iNOS gene expression, increasing iNOS protein expression and producing great amounts of NO, involved in apoptosis.
- Nuclear factor κB would thereby act as a survival or death factor, depending on the pathways involved and the cell conditions. This double role would mean that small activation of NFκB could permit cell death, whereas a great activation would stop it. Activation/inhibition mainly depends on the time curse of cellular and molecular events.
- The JAK-STAT3 pathway would be involved in these effects by modulating nNOS phosphorylation and iNOS expression, the last effect probably by modulating NFκB activity.

To sum up, both isoforms (nNOS and iNOS) and NFκB are involved in the IFNγ-induced apoptosis in chromaffin cells. The iNOS expression in chromaffin cells, shown by the first time in this paper, suggest that, as well as in neurons (Minc-Golomb *et al.* 1996), this enzyme could contribute significantly to the vulnerability of the neural cells to various inflammatory insults and once more support the use of chromaffin cells as a neural model to study molecular mechanisms of neuronal cell death underlying neurodegenerative diseases.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Calcium dependence and effects of NOS inhibitors on NOS activity induced by IFNγ in bovine chromaffin cells.

Supplementary Material and Methods Chemical used.

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Supporting information
Supplementary Material and Methods: Chemical used

Supplementary Figure Legends

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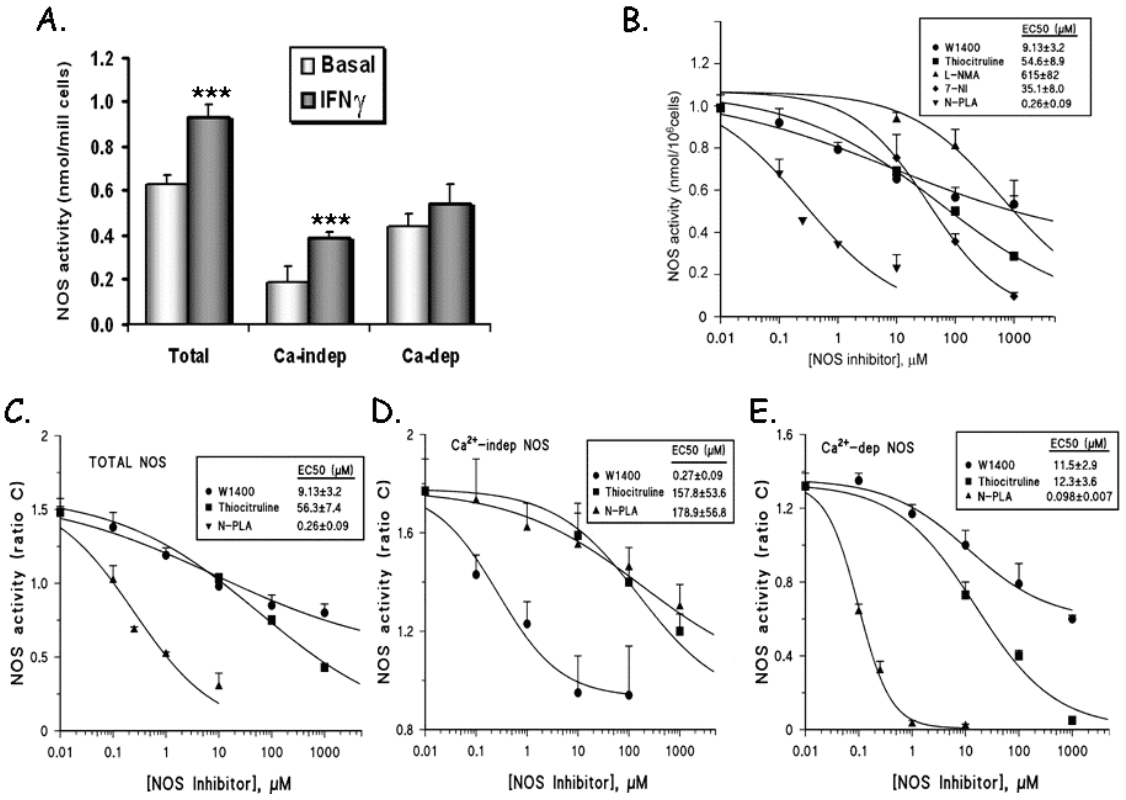
Dulbecco's modified Eagle's medium (GIBCO); LPS, N-(3-aminomethyl)benzyl)acetamidine (W1400), 3-Bromo-7-nitroindazole, S-Methyl-L-thiocitrulline acetate salt, L-NMA, calmidazolium, dexamethasone, glutamate, anti-β-actin, anti-mouse IgG peroxidase-conjugate and anti-rabbit IgG peroxidase-conjugate were from SIGMA Chemical (Madrid, Spain). Oligonucleotide primers were synthesised at Sigma Genosys. IFNγ and TNFα were from Prepotech; N-ω-Propyl-L-arginine (N-PLA) was from Tocris Bioscience (Bristol, UK); 2,3-diaminonaphtalene (DAN) and SN50 were obtained from Calbiochem-Novabiochem Co (La Jolla, CA, USA). Cell Proliferation Kit II (XTT) was from Hoffmann-La Roche Ltd Diagnostics (Basel, Switzerland), Dowex AG50W-X8 resin and Bio-Rad protein assay from Bio-Rad Laboratories S.A. (Madrid, Spain), ECL detection kit from Amersham Biosciences (NJ, USA) and L-[U14C]arginine from New England Nuclear (Perkin Elmer Life Sciences, Boston, Mass.). Anti-phospho-STAT3 (ser 727) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany), anti-Phospho-S847 nNOS from Abcam (Cambridge, UK); rabbit anti-nNOS and mouse anti-iNOS were from BD-Transduction Laboratories (BD-Biosciences, San Diego, CA) and anti-IkBα from Epitomics, Inc. (Burlingame, CA, U.S.A.). RNeasy mini kit and RNase-free DNase were from Qiagen Ltd, Sussex, UK); SuperScript III Reverse Transcriptase from Invitrogen S.A, Barcelona, Spain); Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.); Oligonucleotide against proximal -κB motif (nt 92 a -65) of the rat NOS-2 promoter was from Isogen Life Science, IJsselstein, The Netherlands) and [32P]dCTP from New England Nuclear (PerkinElmer, Inc., Massachusetts, USA). Chemicals and reagents not detailed here were reactive grade products from Merck Sharp & Dohme (Madrid, Spain), Sigma-Aldrich Química, S.A. (Madrid, Spain), Bio-Rad laboratories S.A. (Madrid, Spain) or Boehringer Ingelheim (Barcelona, Spain).

Supplementary Figure

Figure S1: Calcium dependence and effects of NOS inhibitors on NOS activity induced by IFNγ in bovine chromaffin cells.
Cultured bovine chromaffin cells (106/condition) were incubated in the absence (control) or presence of 200 ng/ml IFNγ, in the absence or presence of different NOS inhibitors, for 24h either in a DMEM medium containing 2.5 mM Cl2Ca (total activity) or in a medium without calcium (containing 4 mM EGTA + 10 µM CaZ; calcium-independent activity). Then, supernatants were removed, cells were lysed and NOS activity was measured by quantification the production of [U-14C]L-citrulline from [U-14C] L-arginine as described in Material and Methods. Calcium-dependent NOS activity was calculated as the difference between the amount of synthesized [14C]L-citrulline released in the presence of calcium (total NOS activity) and [14C]L-citrulline formed in the absence of extracellular calcium (calcium-independent NOS activity). NOS activity was expressed as nmol/106 cells/24 h (A-B) or as ratios over the effect-induced by IFNγ in each condition (1.41±0.10, 1.77±0.23 and 1.22±0.13 times their respective basal levels for total, calcium-independent and calcium-dependent NOS activity) (C-E). Fitting of the concentration–response curves for estimation of IC50 values was made by weighted nonlinear regression of minimum squares, using logistic curves. The data are mean ± SEM of three determinations, each performed in duplicate. Statistic in A compares the effect of IFNγ with their specific basal controls (***p<0.001) (one-way ANOVA test).

Figure S1



Signalling mechanisms of glutamate-induced apoptosis in chromaffin cells. Involvement of nNOS but not of iNOS or NFκB

Title: "Signalling mechanisms of glutamate-induced apoptosis in chromaffin cells. Involvement of nNOS but not of iNOS or NFκB"

Short running title: Mechanisms of glutamate-induced apoptosis in chromaffin cells

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Abbreviations: AMPA= a-Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid CA= catecholamines cNOS = constitutive NOS cGMP= 3'-5'-cyclic guanosine monophosphate D-AP5= D(-)-2-amino-5-phosphonopentanoic acid Glu= glutamic acid HEPES = N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid IFNγ = gamma-interferon iGluR = ionotropic glutamate receptor I-κB = nuclear factor κB inhibitor iNOS= inducible NOS KA= Kainic Acid L-NMA = N-methyl L-arginine LPS = lipopolysaccharide MCPG = alpha-methyl-4-carboxyphenylglycine mGluR = metabotropic glutamate receptor NBQX = 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline NF-κB = nuclear factor κB NGF = Nervous growth factor NMDA= N-Metil-D-Aspartato nNOS= neuronal NOS NO = nitric oxide NOS = nitric oxide synthase N-PLA= N-propil-L-arginine P = peroxynitrite SNP = sodium nitroprusside t-ACPD = trans-1-amino-cyclopentane -1,3-dicarboxylic acid TNFα = tumor necrosis factor alfa thiocitrulline = S-methyl-L-thiocitrulline

ABSTRACT

Previous work from our group stated that nitric oxide (NO), stimulated either exogenously or endogenously, via cytokines, induces apoptosis in chromaffin cells by a mechanism involving iNOS, nNOS and NFκB. In this paper the involvement of glutamate as a possible intracellular trigger of NO-mediated apoptosis has been evaluated. We show that chromaffin cells express different ionotropic and metabotropic glutamate receptors, exerting different effects on the regulation of basal and glutamate induced catecholamine (CA) secretion, via NO/cGMP. In addition, we studied the effect of endogenously generated NO, basal and stimulated with glutamate, on apoptosis of chromaffin cells, as well as the possible involvement of nNOS, iNOS and NFκB in the glutamate/NO-induced apoptosis.

Our results show that glutamate agonists are able to induce cell death and apoptosis in bovine chromaffin cells, in parallel to an increase in NO production. Glutamate-induced apoptosis and NO production were reverted by NOS inhibitors and glutamate-antagonists, thereby showing that NO is involved in glutamate-induced apoptosis. Cells were treated with specific NOS inhibitors (1400-W for iNOS, N-PLA for nNOS and 7-NI, L-methyl thiocitrulline and L-NMA, as more unspecific nNOS inhibitors). Under basal conditions, iNOS inhibitors did not have any effect on apoptosis, whereas nNOS inhibitors induced apoptosis, indicating a neuroprotective effect of constitutive nNOS-generated NO. In contrast, glutamate-induced apoptosis was strongly reverted by nNOS inhibitors and weakly by iNOS inhibitors, thus indicating the nNOS involvement in glutamate-mediated apoptosis. These results were confirmed by the fact that nNOS expression, but not iNOS, is specifically activated by glutamate. Additional evidence on the mechanisms involved in glutamate-induced apoptosis was assessed through activation of NFκB. NFκB is a transcriptional factor that stimulates iNOS expression. NFκB was not activated by glutamate, but by nNOS inhibitors. However, our results seem to suggest the participation of PKG, PKA, PKC and MAPKs pathways in glutamate-mediated nNOS activation in chromaffin cells and point out the involvement of both PKA and PKC signalling pathways in the apoptotic effect of glutamate.

Taken all together, these data suggest that endogenous NO plays a dual role on chromaffin cell death and survival. Under basal conditions, these cells express nNOS, producing physiological and cytoprotective amounts of NO, and blocking NFκB translocation to the nucleus. Glutamate-induced stimulation would induce nNOS activation, leading to high and toxic concentrations of NO, involved in apoptosis.

Keywords: Glutamate, Nitric Oxide, Nitric Oxide Synthase, Cell death, Chromaffin cells, Apoptosis, Signal transduction, NFκB.

INTRODUCTION

Nitric oxide (NO) is a cellular messenger playing very important roles in the nervous system as a regulator of neurotransmission, as well as in pathological events underlying neurotoxicity and neurodegeneration. Endogenous NO is synthesized from L-arginine by three isoforms of NO synthase (NOS). Two isoforms are expressed constitutively, the neuronal (nNOS or type 1) and the endothelial (eNOS or type 3). The third isoform is induced under pathological conditions and inflammation (iNOS or type 2), and is able to produce large amounts of NO for a long period of time (Bredt, 1990). All three isoforms are found in the CNS. The induction of a high output system for NO in response to cytokines, (Liu et al., 2002) or a massive production of NO following accumulation of glutamate (Nakamura et al. 2007), can result in cell death and in pathological events underlying neurotoxicity and neurodegeneration. In fact, inhibition of nNOS and iNOS activity ameliorates the progression of disease pathology in animal models of different neurodegenerative diseases (Calabrese et al. 2007).

In bovine chromaffin cells, previous works from our group indicated that NO, either exogenously administered through NO donors, or endogenously generated via cytokines, induced apoptosis in chromaffin cells (Vicente et al. 2006) by a mechanism involving mitochondria (Pérez-Rodríguez et al. 2007), as well as nNOS and iNOS isoforms and the transcriptional factor NFκB (Pérez-Rodríguez et al. 2009). Therefore, cytokines might be one of the possible external sources of NO induced apoptosis in chromaffin cells. However, other molecules could also be responsible for yielding of this apoptotic endogenous molecule. Among these, the excitatory neurotransmitter glutamate could be a good candidate, as it has been described a deathly relationship between glutamate overactivation and NO (Brown 2010).

In regards to glutamate in chromaffin cells, previous work of our research group demonstrated that this neurotransmitter may be involved in the regulation of catecholamine (CA) secretion, by interacting specifically with different glutamate receptor subtypes: NMDA, AMPA, KA and t-ACDP (González et al. 1998; Arce et al. 2004). Thus, the role of glutamate as a potential neurosecretory regulator in this tissue has been established. However, glutamate could also have a prodeath function in these cells, but the role of glutamate as a possible excitotoxic agent in chromaffin cells has not yet been studied.

In this work we try to assess the role of glutamate in chromaffin cell by studying 1) Its possible apoptotic effect on chromaffin cells and the types of glutamate receptors involved in this effect, 2) The role of intracellular NO and NOS isoforms, as well as of the transcription factor NFκB, in glutamate-mediated apoptotic cell death of chromaffin cells and 3) The possible signalling pathways underlying these effects.

MATERIAL AND METHODS

1) Chemicals

Dulbecco's modified Eagle's medium, foetal calf serum, HEPES and RNAse A were from GIBCO (BRL, UK), collagenase from Clostridium histolyticum (EC 3.4.4.19) was supplied by Boehringer Mannheim S.A. (Barcelona, Spain). The NOS inhibitor S-metyl-L-thiocitruline hydrocloride (thiocitrulline) was obtained from Tocris Cookson (Bristol, UK). Antibiotics, cytosine arabinoside, 8-fluoro-desoxiuridine (FDU), neutral red and propidium iodide were from SIGMA Chemical (Madrid, Spain). Amphotericin B was from ICN Ibérica S.A. (Barcelona, Spain). The fluorogenic substrate peptide Ac-DEVD-amc was supplied by BD PharMingen International (Becton Dickinson Co., USA). Peroxynitrite and carboxy-PTIO were purchased in Alexis Biochemicals (Lausen, Switzerland) and 2,3-diaminonaphtalene (DAN)] were from Calbiochem-Novabiochem Co (La Jolla, CA, USA) . All other chemicals were reactive grade products from Merck (Darmstadt, Germany). Fluorescent probes 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and tetramethyl rhodamine methyl ester (TMRM) were from Molecular Probes (Eugene, OR, USA). Anti–Bcl-x (sc-634) and anti–caspase-9 (sc-7885) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–cytochrome c monoclonal antibody and caspase-3 substrate Ac-DEVD-AMC, were from Pharmingen (San Diego, CA). Radiochemicals were from ICN (Irvine, CA). Other reagents were from Sigma Chemical Co. (St. Louis, MO) or Boehringer (Mannhein, Germany).

2) Methods

Chromaffin cell primary culture and drug treatments

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Pérez-Rodríguez et al. (2009). Briefly, glands were incubated with Ca2+-free Locke containing 0.1% protease (Sigma, St Louis, MO, USA) for 20 min (2·10 min). Medulla was detached from cortex, submitted to mechanical disgregation, and further incubated with 0.1% collagenase (Worthington) in Ca2+-free Locke for 25 min in a shaker. After digestion, the solution was filtered through a 190 lm pore nylon membrane, and chromaffin cells purified through a series of 10 min centrifugations (1x 180 g, 6x 50 g) using a 4% albumin gradient in the last one. Cell viability, purity checking and cell plating and treatments were performed as described (Vicente et al. 2006) and used 3-7 days after plating.

Measurement of nitrite production

Nitrites were determined with the spectrofluorimetric method of Misko et al. (1993) with minor modifications as described by Vicente et al. (2006). This method is based on the measurement of the fluorescent product 1-(H)-naphthotriazole formed by the reaction of nitrites with 2,3-diaminonaphtalene (DAN) under acidic conditions.

Samples were calibrated with a standard curve of freshly prepared nitrites and results were expressed as arbitrary fluorescence units (AFU).

Measurement of intracellular levels of cGMP

Cells were stimulated for 10min with the appropriate secretagogues, as indicated. Incubations were perfor-

Primer	Sequence
GA3PDH forward	5'-CACAGTCAAGGCAGAGAACG-3'
GA3PDH reverse	5'-TACTCAGCACCAGCATCACC-3
nNOS forward	5'-TGATCATCTCTGACCTGATTCG-3'
nNOS reverse	5'-ATAGCTCAGGTCCACCAAGG-3'
eNOS forward	5'-GCGATGTCACTATGGCAACC-3'
eNOS reverse	5'-CGTGATAGCGTTGCTGATCC-3'
iNOS forward	5'-AGCATGTGTTCACCATGAGG-3'
iNOS reverse	5'-GCAGGTAGCTTGGAGCTTGG-3'
NMDA, subunit 1; GRIN1 f	5'-ACCCGCATGTCCATCTACTC-3'
NMDA, subunit 1; GRIN1 r	5'-TCCAGCTGTAGACACGCATC-3'
NMDA, subunit 2A; GRIN2A f	5'-GACTCACCCAAAAGAGCTGC-3'
NMDA, subunit 2A; GRIN2A r	5'-CTCTTTCCCCTGAAAGGACC-3'
NMDA, subunit 2B; GRIN2B f	5'-TTGTCAACAAGATTCGCAGC-3'
NMDA, subunit 2B; GRIN2B r	5'-CTTAGAATCCCCATCGTCCA-3'

Primer	Sequence
AMPA, subunit 1; GRIA1 f	5'-GGGATGATTCGAGTGAGGAA-3'
AMPA, subunit1; GRIA1 r	5'-CACCTTCATGGTGTCACAGG-3'
AMPA, subunit 2; GRIA2 f	5'-CCATTTGTCATTCAGATGCG-3'
AMPA, subunit 2; GRIA2 r	5'-TGGACAAGCCTCTGTCACTG-3'
KA, subunit 1; GRIK1 f	5'-TAAAATCAAAATCCGCCAGC-3'
KA, subunit 1; GRIK1 r	5'-TAAGGATCTCAGCGGCTGTT-3'
KA, subunit 2; GRIK2 f	5'-TCATGCAGCAAGGTTCTGAG-3'
KA, subunit 2; GRIK2 f	5'-TGTCAGAAAGGCGGCTAAGT-3'
mGluR1; GRM1 f	5'-GCCGCTCCAACACTTTACTC-3'
mGluR1; GRM1 r	5'-CACCTGTCTTCCACCTGGTT-3'
mGluR5; GRM5 f	5'-GTTTGCACAGGAGAACAGCA-3'
mGluR5; GRM5 r	5'-ACGCTCATAGAGCTCACCGT-3'
mGluR2; GRM2 f	5'-AGTGATTACCGGGTGCAGAC-3'
mGluR2; GRM2 r	5'-GTGGCTGACCACGTTCTTTT-3'

med in the presence of 0.5 mmol/L IBMX in order to prevent cGMP degradation. The medium was removed and the cells lysed with 200 µl of 0.4 mol/L $HClO_4$. These cell lysates were neutralized with 1 mol/L KOH, centrifuged in an Eppendorf centrifuge, and the supernatants used for assays of cGMP. Intracellular cGMP measurements were performed by using the specific radioimmunoassay kits from Amersham, following the manufacturer's instructions.

Measurement of cell viability by the XTT test:

Changes in cell viability induced by glutamate, in the presence and absence of NOS inhibitors, were measured by the XTT assay as described (Figueroa et al., 2006). Results were expressed as ratios over the respective controls (untreated cells).

Flow cytometric analysis of apoptosis

Measurement of glutamate-induced apoptosis, both in the presence and in the absence of NOS inhibitors, was carried out by flow cytometry as described (Pérez-Rodriguez et al., 2009; Figueroa et al., 2006). Results were expressed as ratios over the respective controls (not-treated cells).

Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts were isolated by a modified procedure based on the method of Andrews and Faller (1991) as previously described (Pérez-Rodríguez et al., 2007). Aliquots of extracts were analyzed for protein content using the Bio-Rad protein reagent.

Electrophoretic mobility shift assays (EMSAs)

The oligonucleotide sequence corresponding to the NFκB site was the proximal κB motive (nucleotides -92 to -65) of the rat NOS-2 promoter (tcga 5' CCAACTGGGGACTCTCCCTTTGGGAACA 3'
and tcga 5'TGTTCCCAAAGGGAGAGTCCCCAGTTGG 3'). EMSA assays were performed as described (Pérez-Rodríguez et al, 2009).

Western blot analysis

Western blots were carried out as described by Pérez-Rodríguez et al. (2009). Band intensities were measured on a densitometric scanner, and normalized with respect to β-actin expression.

RT-PCR analysis

RNeasy Mini Kit was used for total RNA isolation. Reverse transcription (RT) was carried out for one hour at 55ºC with oligodeoxythymidylate primer using 5 µg of total RNA from each sample for complementary DNA synthesis.

Semiquantitative and real time quantitative PCR were performed in order to determine the levels of NOS isoforms, iGluRs and mGluRs, as well as housekeeping GAPDH and β-actin mRNAs by using the following specific primers synthesized at Sigma-Aldrich Co., as shown in the table.

Real-time PCR

The SYBR Green PCR Master Mix (Applied Biosystems) and the 7900 HT Fast Real-Time PCR system (Applied Biosystems) were used for detecting the real-time quantitative PCR products of reverse-transcribed cDNA samples, according to the manufacturer's instructions. q-PCR conditions were: 95ºC (10 min) followed by 40 cycles of 15 seconds at 95ºC and annealing for 1 minute at 60ºC. Three independent quantitative PCR assays were performed for each gene and measured in triplicate. Three no-template controls (NTCs) were run for each quantitative PCR assay, and genomic DNA contamination of total RNA was controlled using RT minus controls (samples without the reverse transcriptase).

Semiquantitative PCR

Conventional PCR amplifications were conducted in a 25 µl solution containing 1x PCR buffer, 0.2mM dNTP mix, 1.5 mM magnesium chloride, 400 nM of each primer and 1 U of DNA polymerase and 2 µl of cDNA template, corresponding to 5 µg total RNA in a 20 µl final volume. Negative control of amplification was performed with 2 µl of water instead of cDNA template. Amplification conditions were: 2 min at 95ºC, 11 cycles of 30 s at 95ºC, 30 s at 61ºC, decreasing 0.5ºC every cycle, and 20 s at 72ºC, followed by 23 cycles of 30 s at 95ºC, 30 s at 55.5ºC, and 20 s at 72ºC, and a final extension of 2 min at 72ºC. Reactions were carried out in a thermal cycler. 10 µl of the PCR products were resuspended in 6X loading buffer (30% glycerol, 0.5 µg/ml BrEt) and electrophoresed through 1.5% agarose in 0.5X TBE buffer (45 mM Tris-borate; 1 mM Na2EDTA pH 8·0) with 0.5µg/ml BrEt for 1.5 h.

Statistics

Data were expressed as means±SEM values of three or four independent experiments with different cell batches, each of which was performed in duplicate or triplicate. Statistical comparisons were performed by using one-way analysis of variance (ANOVA), followed by Holm–Sidak's post test when the analysis of variance was significant. Differences were accepted as significant if $P< 0.05$ or less. Statistical analyses were carried out using Sigmastat software (Systat Software, Inc., Germany).

RESULTS

1. Chromaffin cells express mRNA of the main ionotropic and metabotropic glutamate receptors, some of them in a constitutive fashion and others under glutamate induction

Previous work of our research group demonstrated that glutamate may be involved in the regulation of catecholamine (CA) secretion by interacting specifically with different glutamate receptor subtypes: NMDA, AMPA, KA and t-ACDP (González et al., 1998; Arce et al., 2004). In order to further characterize the expression of the different types of ionotropic and metabotropic glutamate receptor (iGluRs and mGluRs) in chromaffin cells, as well as to gain a further insight into the subunit composition of the different iGluR isotypes, polymerase chain reaction (semi-quantitative for Figure 1A and quantitative for Figures 1B-C) experiments were performed for the different subtypes of iGluRs subunits and some mGluRs subtypes.

Semiquantitative RT-PCR experiments demonstrated the presence of specific mRNAs of NMDA subunit 1A (GRIN1A) and KA subunit 2 (GRIK 2) (Figure 1A). Data also showed a mild presence of AMPA subunit 2 (GRIA 2) and KA subunits 1 (GRIK 1) mRNAs, but not of NMDA subunit 2A and 2B (GRIN 2A, 2B) or of AMPA subunit 1 (GRIA 1) (Figure 1A). Treatment with 1 mM glutamate was found to increase the expression of NMDA 1A, AMPA 2 and KA 2 subunits mRNAs, and to induce the expression of NMDA 2A subunit mRNA (Figure 1A), yet not that of NMDA 2B, AMPA 1 and KA 1 (data not shown). Semiquantitative RT-PCR experiments were also able to detect the expression of mGluR1 and mGluR5, but not that of mGluR2 mRNA (Figure 1B). The expressions of mGluR1 and 5 were increased by chromaffin cell treatment with 1 mM glutamate (Figure 1B). All in all, our data indicate that the main iGluRs (NMDA, AMPA and KA) and specific Class I excitatory mGluRs (mGluR1 and 5) are expressed in primary cultures of bovine chromaffin cells.

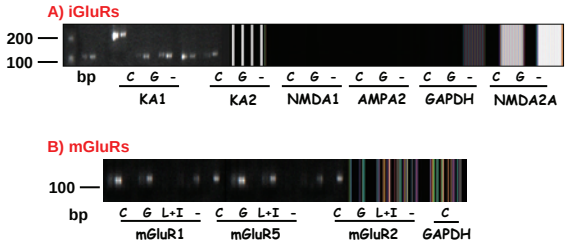


Figure 1. mRNA expression and regulation of different ionotropic (A) and metabotropic (B) glutamate receptors in bovine chromaffin cells. Bovine chromaffin cells were incubated for 6 h in the absence (C) and in the presence of 1 mM glutamate (G) or LPS 10 µM plus IFNγ 10 nM (L + I). mRNA was isolated and treated with appropriate set of primers for different ionotropic glutamate receptor subunits (iGluRs, A), metabotropic receptors (mGluRs, B) or GAPDH (B), as a control. Conventional RT-PCR were carried out as described in Materials and Methods.

2. Activation of glutamate receptors expressed in chromaffin cells increases catecholamine secretion in a manner dependent on nitric oxide production and NOS activation.

In order to have further evidence about the possible involvement of these glutamate receptors on CA secretion by chromaffin cells, we surveyed the effect of agonists of iGluRs and mGluRs on catecholamine secretion in the absence and presence of thiocitrulline, a known nNOS inhibitor (Vicente, González, and Oset-Gasque 2002; Pérez-Rodríguez et al. 2009).

Stimulation of chromaffin cells with 10-100 µM glutamate agonists without thiocitrulline elicited a dose-dependent increase of CA secretion, the order of potency being AMPA>NMDA=t-ACPD>Glu>KA (Figure 2A). Maximal responses were about half of those obtained with 10 µM nicotine (Figure 2B).

To ascertain whether endogenous NOS-generated NO could be involved in the secretory mechanism induced by glutamate agonists, we also studied the effect of thiocitrulline on CA secretion induced by agonists of glutamate receptors. Figure 2A shows that, with the exception of KA-induced CA secretion, thiocitrulline was able to almost completely abolish the effect of glutamate agonists on CA secretion, (Figure 2A). These inhibitory effects of thiocitrulline were higher than those obtained for the secretory effect induced by nicotine (25-50% inhibition), which was not observed for secretory effect of high 56 mM KCl (Figure 2B).

In parallel to the increase in CA secretion (Figure 2C), glutamate agonists were also able to increase NO_2^- and cGMP levels (Figures 2D and 2E), both effects inhibited by 0.5 mM Thiocitrulline.

3. Glutamate agonists induce apoptosis in parallel to NO production

Previous work from our group stated that chromaffin cells could undergo NO- mediated apoptosis under cytokine-stimulation (Pérez-Rodríguez et al., 2009). Also, results above exposed suggested that NO mediates CA secretion under glutamate agonist induction. We wondered whether glutamate, known to cause excitotoxicity, could cause apoptosis in chromaffin cells and whether this effect could be mediated by NO. Cells were challenged with glutamate and glutamate agonists for 24 h at concentrations 0.1-10 mM. Glutamate triggered a concentration-dependent increase in apoptotic cell death (Figure 3A), with a EC50 of 2.09±0.06 mM (Figure 3A, inset). In order to assess which glutamate agonists mediated apoptosis, we challenged the cells with specific glutamate antagonists. The apoptotic effect of 1 mM glutamate was specifically reverted by 200 µM concentrations of NBQX, D-AP5 and MCPG (Figure 3B.

To go further into the participation of different subtypes of glutamate receptors in the apoptotic effect of glutamate in chromaffin cells, cells were challenged with 0.1-1 mM concentrations of different ionotropic (NMDA, AMPA and KA) and metabotropic (t-ACPD) glutamate receptor agonists, alone or in specific combinations. Results in Figure 4A-B show

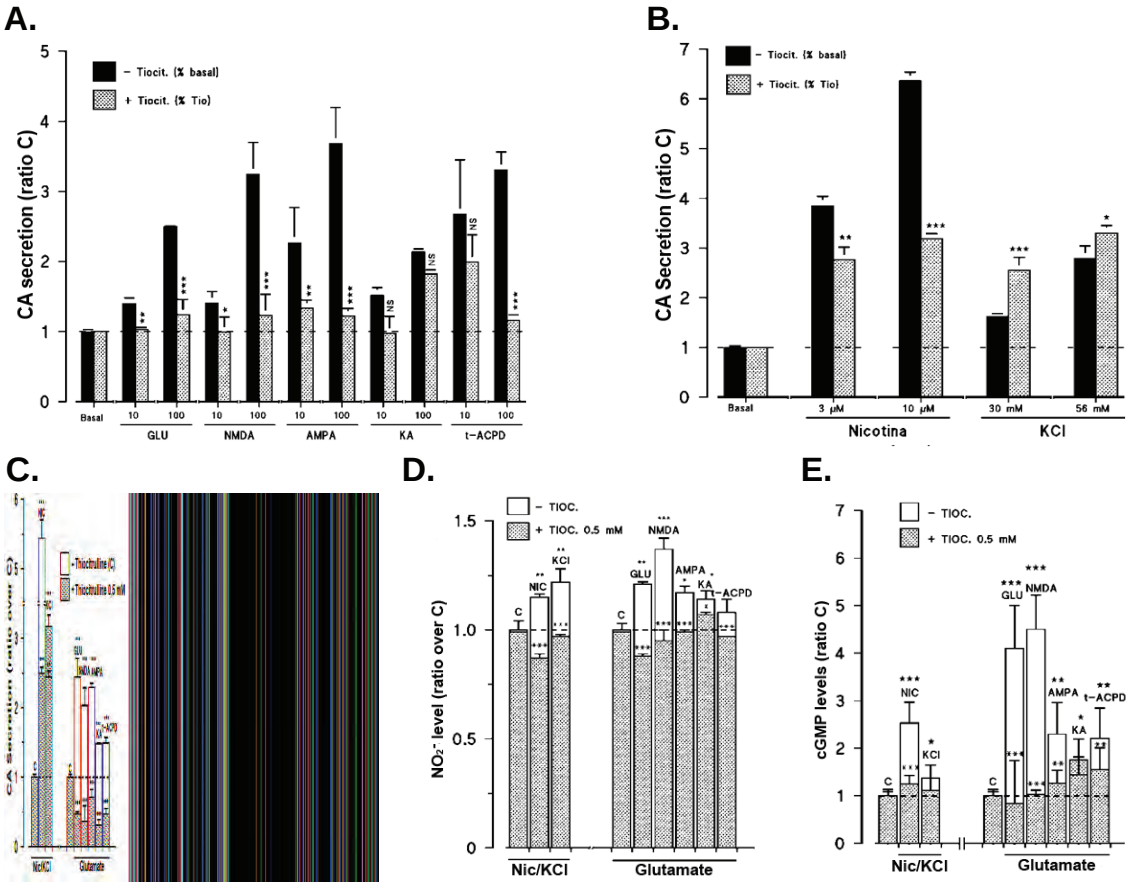


Figure 2. Effect of thiocitrulline on CA secretion (A-C), NO levels (D) and cGMP levels (E) induced by glutamatergic agonists. Cells were incubated simultaneously with thiocitrulline and different glutamate agonists, as indicated in Material and Methods. NMDA agonist assays were performed on a $MgCl_2$ free medium. Results are expressed as ratios over their respective controls and are mean ± SEM values, and compare the effect of each agonist versus its correspondent basal in the absence or presence of 0.5 mM thiocitrulline. Basal CA secretion =4.01±0.3 % in the absence, and 6.4±0.5 % in the presence of thiocitrulline (n = 6). Basal NO_2^- and cGMP levels were 0.58±0.04 nmol/mill cells and 3.34 pmol/mill cells, respectively, in the absence of thiocitrulline (white bars) and of 0.52±0.02 nmol/mill cells or 2.53±0.2 pmol/mill cells, respectively, in the presence of thiocitrulline (ruled lines) *p < 0.05; **p < 0.01; ***p < 0.001 (one way ANOVA test).

that all glutamate agonists were able to cause apoptosis in a dose-dependent manner. The highest records, 4 times basal levels, were reached with a mix of 1mM NMDA+AMPA (Figure 4B), showing a synergic effect on apoptosis induction.

Similar studies were carried out measuring nitrite production at a concentration of 0.5mM glutamate or glutamate agonists, in the presence and absence of specific glutamate antagonists (MK-801and D-AP5 for NMDAR; NBQX for AMPAR and MCPG for mGluR1-5). All glutamate agonists increased NO production, particularly non-NMDA type receptors (AMPA and KA) that were 1.8 fold times higher than the basal rate (Figure 4C). The NMDA-type receptor antagonists MK-801 and D-AP5 reverted the NO_2^- levels increase induced by glutamate and NMDA, but not that for AMPA. NBQX, the AMPA-type receptor antagonist reverted NO_2^- level increases induced by glutamate and AMPA stimulation, but not that for NMDA. MCPG specifically reverted the increase in NO_2^- levels induced by glutamate and t-ACPD (Figure 4C).

4. nNOS plays an anti-apoptotic and neuroprotective role in basal and glutamate-stimulated conditions, whereas iNOS is not involved

We wanted to further investigate the involvement of endogenously generated NO under basal and glutamate stimulated conditions, and the specific NOS isoforms mediating these effects on apoptosis. Therefore, we tried to find out any existing parallelism between NO production and cell death under both previously mentioned conditions. We also assessed the involvement of the different NOS isoforms by using specific inhibitors. Cells were treated for 24h with NOS inhibitors at concentrations ranging from 0.1 to 1000 µM, in the presence and absence of 2 mM glutamate, and apoptosis and nitrite production were checked. The NOS inhibitors employed were: W1400, specific iNOS inhibitor; N-PLA, specific nNOS inhibitor; Thiocitrulline, L-NMA and 7-NI, unspecific nNOS inhibitors.

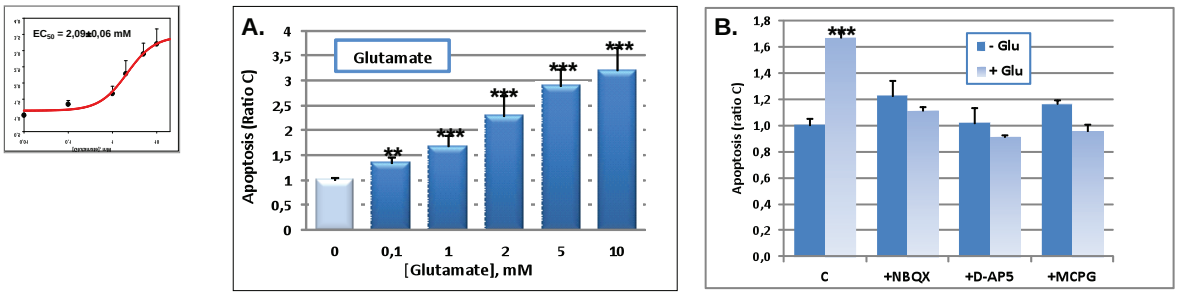
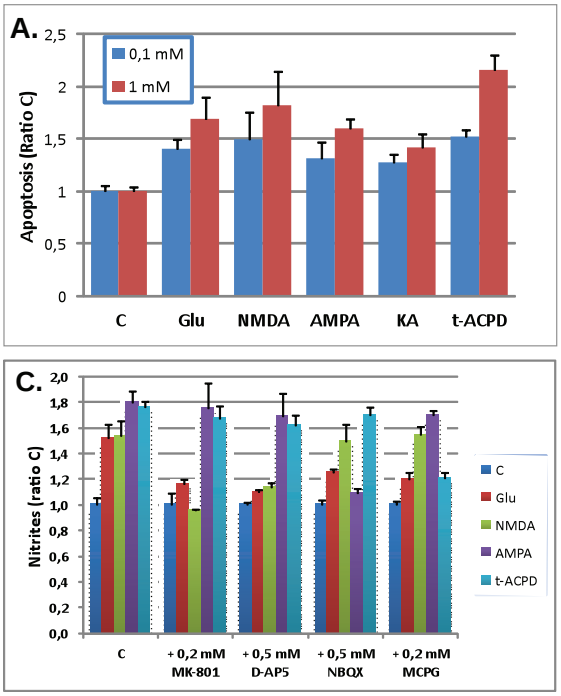


Figure 3. Dose-response effects of glutamate (A) and glutamatergic antagonists (B) on apoptosis in chromaffin cells. Bovine chromaffin cells were incubated for 24 h with increasing concentrations of glutamate (A) or with 1 mM glutamate in the absence and presence of 200 µM concentrations of indicated receptor antagonists (B), and apoptosis was measured as indicated in Material and Methods. Data were expressed as ratios over control and are mean ± SEM values obtained from three experiments each one performed in quadruplicate. Basal apoptosis = 4.2±045 %. *p < 0.05; **p < 0.01; ***p < 0.001 (one way ANOVA test).



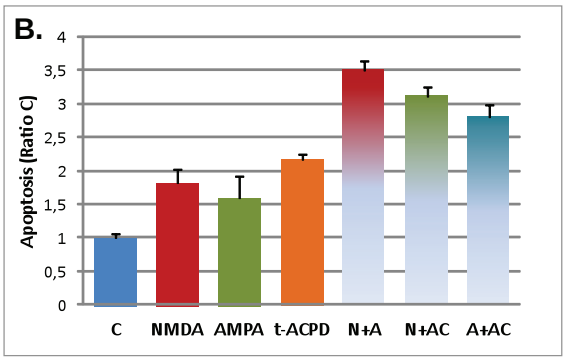
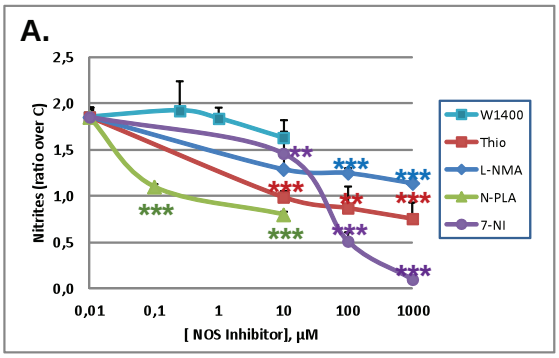


Figure 4. Effect of glutamate receptor agonists on apoptosis (A-B) and NO levels (C) in chromaffin cells. Bovine chromaffin cells were incubated for 24 h with the 0.1-1 mM (A) or 1 mM (B-C) concentrations of glutamate agonists, in the absence and presence (C) of shown concentrations of antagonists. Apoptosis (A-B) and NO2- levels (C) were measured as indicated in Material and Methods. Data were expressed as ratios over control and are mean ± SEM values obtained from three experiments each one performed in quadruplicate. Basal apoptosis = 3.9±0.52 % M1, NO2- basal = 0.68 ± 0.08 nmol/106cells. *** Compare the effect of different treatments versus C and °°° compare the effect of indicated antagonists over the effect produced by the agonist alone. *p < 0.05; **p < 0.01; ***p < 0.001 (one way ANOVA test).



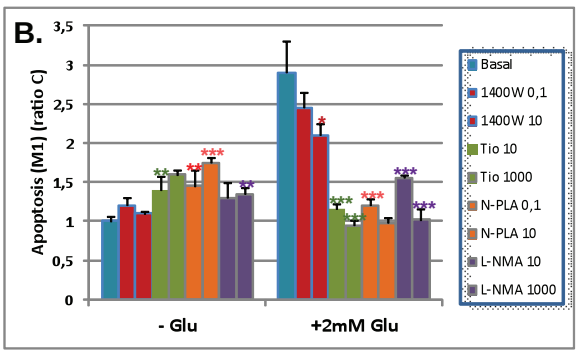


Figure 5. Effect of different concentrations of NOS inhibitors on the increase in NO levels induced by 2 mM glutamate (A), and on basal and glutamate-induced apoptosis (B) in bovine chromaffin cells. Bovine chromaffin cells were incubated for 24 h with the indicated concentrations (µM) of NOS inhibitors (W-1400, thiocitrulline, L-NMA, 7-NI, N-PLA). A) In the presence of 2 mM glutamate, and nitrite formation was measured as described in Materials and Methods. B) In the absence (-Glu) and presence (+Glu) of 2 mM glutamate, and apoptosis was measured as described in Materials and Methods. Data were expressed as ratios over control and are mean ± SEM values obtained from three experiments each one performed in triplicate. [NO2-] basal = 0.75 ± 0.07 nmol/106 cells; Basal apoptosis = 4.1±037 %. *p < 0.05; **p < 0.01; ***p < 0.001 (one way ANOVA test).

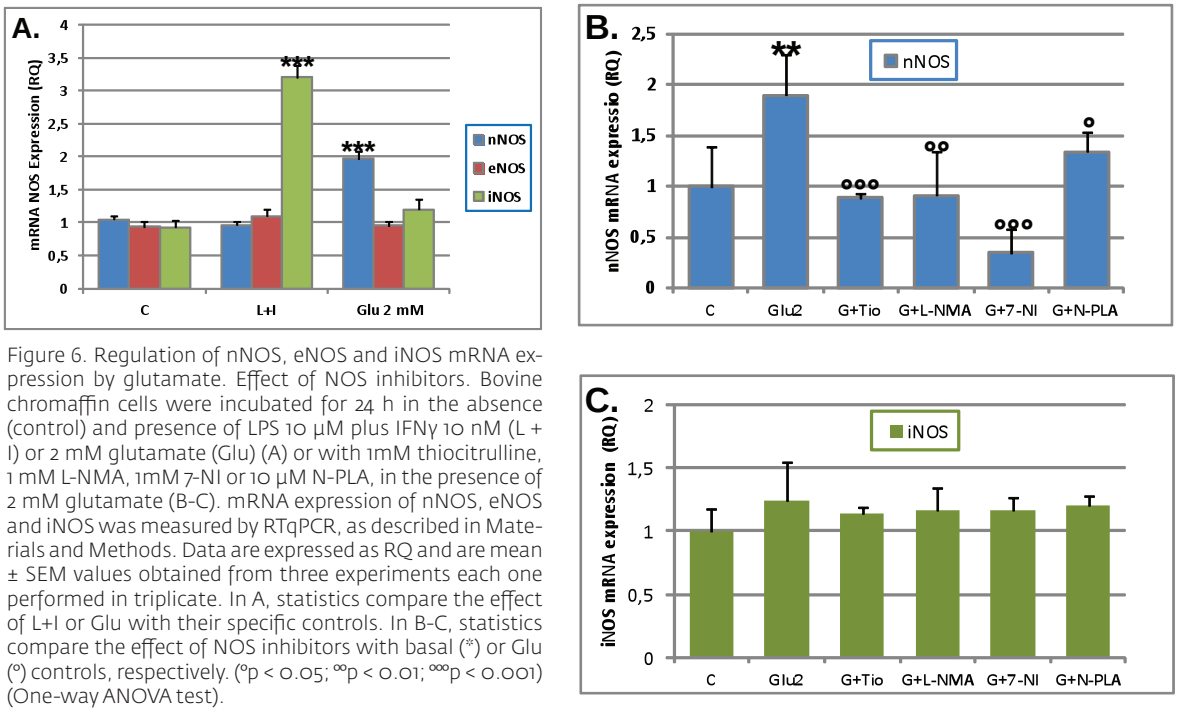


Figure 6. Regulation of nNOS, eNOS and iNOS mRNA expression by glutamate. Effect of NOS inhibitors. Bovine chromaffin cells were incubated for 24 h in the absence (control) and presence of LPS 10 µM plus IFNγ 10 nM (L + I) or 2 mM glutamate (Glu) (A) or with 1mM thiocitrulline, 1 mM L-NMA, 1mM 7-NI or 10 µM N-PLA, in the presence of 2 mM glutamate (B-C). mRNA expression of nNOS, eNOS and iNOS was measured by RTqPCR, as described in Materials and Methods. Data are expressed as RQ and are mean ± SEM values obtained from three experiments each one performed in triplicate. In A, statistics compare the effect of L+I or Glu with their specific controls. In B-C, statistics compare the effect of NOS inhibitors with basal (*) or Glu (°) controls, respectively. (°p < 0.05; °°p < 0.01; °°°p < 0.001) (One-way ANOVA test).

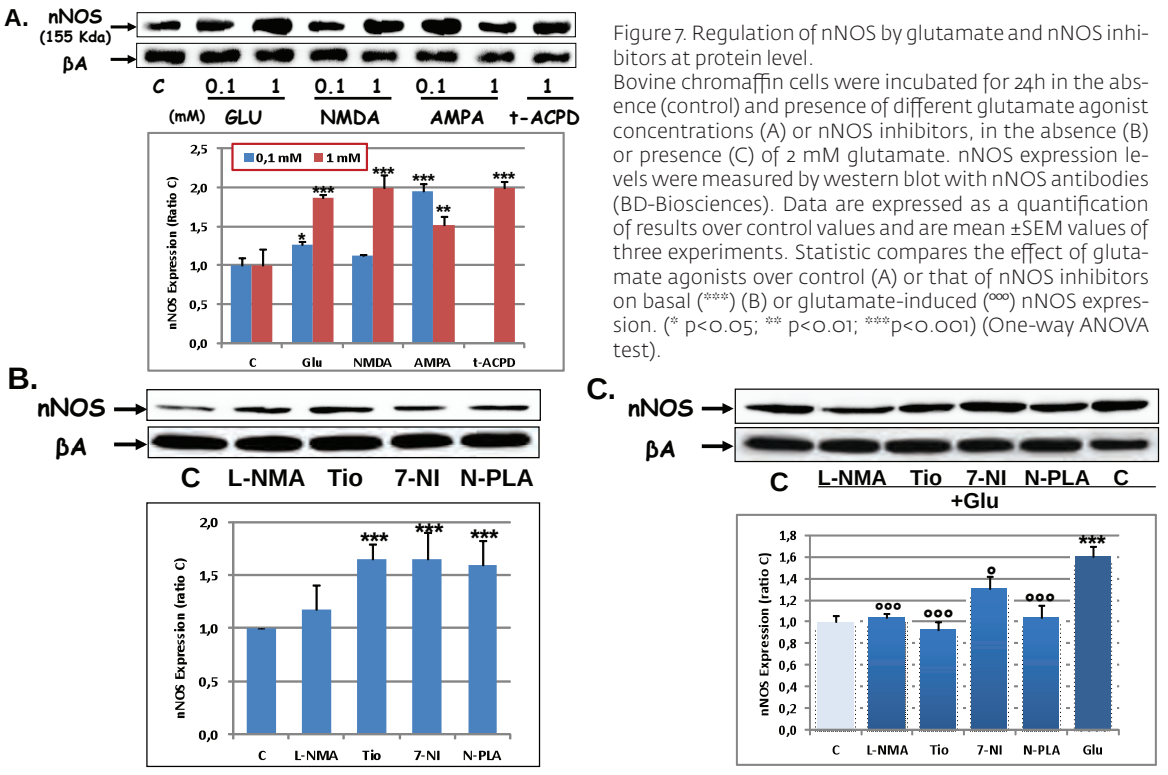


Figure 7. Regulation of nNOS by glutamate and nNOS inhibitors at protein level.
Bovine chromaffin cells were incubated for 24h in the absence (control) and presence of different glutamate agonist concentrations (A) or nNOS inhibitors, in the absence (B) or presence (C) of 2 mM glutamate. nNOS expression levels were measured by western blot with nNOS antibodies (BD-Biosciences). Data are expressed as a quantification of results over control values and are mean ±SEM values of three experiments. Statistic compares the effect of glutamate agonists over control (A) or that of nNOS inhibitors on basal (***) (B) or glutamate-induced (°°°) nNOS expression. (* p<0.05; ** p<0.01; ***p<0.001) (One-way ANOVA test).

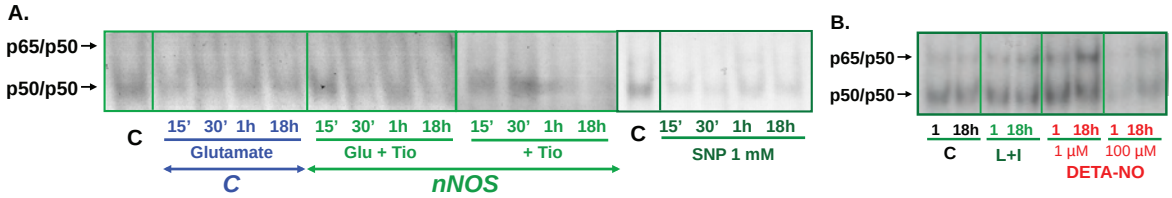


Figure 8. Effect of glutamate and different NO modulators on NFkB translocation to the nucleus.
Chromaffin cells were challenged for 15 and 30 minutes, and 1 and 18 hours, (A) with 2 mM glutamate and 1 mM thiocitrulline, alone or combined, or in the presence of 1 mM SNP; (B), with 10 µM LPS plus 10 nM IFNγ (L+I) or indicated NO donor (DETA-NO and SNP) concentrations. Nuclear extracts were isolated and electrophoresed in an electrophoretic mobility shift assay (EMSA), as described in Materials and Methods. A-C show representative experiments out of three.

4.1 nNOS inhibitors reduce glutamate-induced increase in NO levels and apoptosis, but iNOS inhibitors exert no effect.

As shown in Figure 5A, 0.5-10 µM concentrations of the specific iNOS inhibitor W-1400 (IC50 for total NOS = 9.13±3.2 µM (Pérez-Rodríguez et al., 2009), did not significantly modify nitrite production induced by glutamate. In contrast, nNOS inhibitors lowered nitrite production induced by glutamate in a dose-dependent manner. The power of the different inhibitors at a concentration of 10 µM was as follows N-PLA>Thiocitrulline>7-NI=L-NMA. N-PLA, a specific nNOS inhibitor, (IC50 for total NOS = 0.26±0.09 µM (Pérez-Rodríguez et al., 2009), showed the most powerful blockage of basal NO with a percent inhibition of about a 70%. At concentrations higher than 1 mM, the unspecific nNOS inhibitors, thiocitrulline and 7-NI, inhibited NO production between 70-95%.

Parallel to NO production, we looked into the effect of these NOS inhibitors on basal and glutamate-induced apoptosis in chromaffin cells. Cells were incubated for 24h with NOS inhibitors at two different concentrations, and apoptosis was checked by flow cytometry. W-1400 and N-PLA were used at concentrations 0.1 and 10 µM, and Thiocitrulline and L-NMA were studied at a concentration of 10µM and 1 mM, which had been previously described as concentrations for specific nNOS inhibition.

As observed in Figure 5B, W-1400 did not exert any effect either on basal nor glutamate-induced apoptosis, whereas nNOS specific inhibition by N-PLA, Thiocitrulline and L-NMA, increased basal apoptosis at 1.5-2 fold times the basal levels and inhibited almost completely glutamate-induced apoptosis in parallel to its NO production inhibitory capacity.

4.2 Glutamate mainly induces nNOS expression in chromaffin cells, both at mRNA and protein levels.

In order to deepen into the study of NOS isoforms involved in glutamate-induced apoptosis in chromaffin cells, we surveyed the effect of glutamate, as well as that of different NOS inhibitors, on nNOS, iNOS and eNOS expression, at both protein and mRNA levels.

Quantitative polymerase chain reaction (RTqPCR) experiments were performed to quantify nNOS, iNOS and eNOS mRNA expression. Our data from Figure 6A indicate that, while the main isoform upregulated by cytokines is iNOS, the main isoform regulated by glutamate in chromaffin cells is nNOS, thus confirming our previous results. In Figures 6B-C, we present data on the regulation of nNOS (B) and iNOS (C) mRNA expression, by different NOS inhibitors. Data show that, while the induction of nNOS by glutamate is reverted by all tested nNOS inhibitor, these compounds do not significantly affect iNOS mRNA expression.

These results were confirmed at protein level by western blot experiments. As shown in Figure 7A, glutamate and their main agonists were able to induce a significant increase in nNOS expression in a concentration-dependent manner. Moreover, nNOS inhibitors enhance nNOS expression (Figure 7B), but they were able to inhibit the increase in nNOS expression induced by glutamate (Figure 7C).

5. Signalling mechanisms involved in apoptosis induced by glutamate in chromaffin cells

5.1 Neither glutamate nor NO donors can activate NFκB

Previous results from our group stated the apoptotic effect of cytokines in chromaffin cells, showing an induction of iNOS expression mediated by NFκB activation (Perez-Rodriguez et al, 2009). Therefore, we tested the effect of glutamate and nNOS inhibitors on NFκB translocation to the nucleus, under basal conditions and in the presence of glutamate. Chromaffin cells were challenged for 15min, 30min, 1h and 18h, with 2mM glutamate, in the presence or absence of 1 mM Thiocitrulline, and with 1mM SNP, a NO donor, for comparative purposes.

As seen in Figure 8A, glutamate reduces NFκB translocation to the nucleus at any times (Figure 8A). This result was similar to that induced by

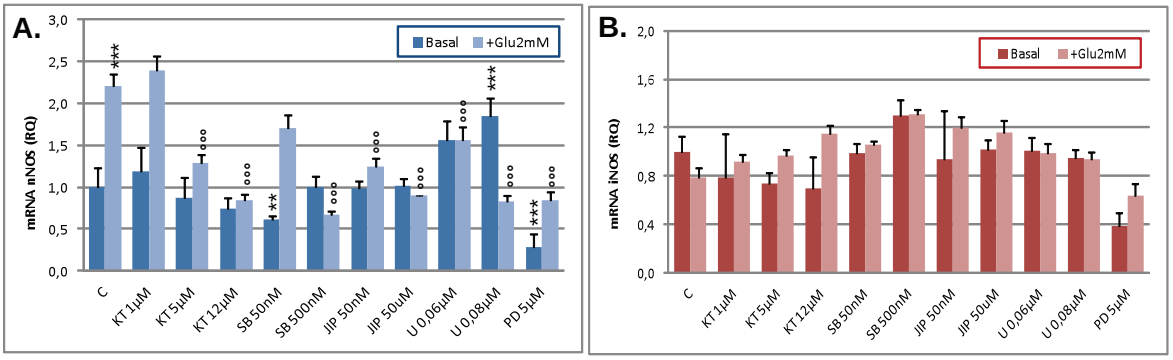


Figure 9. Effect of different protein kinase inhibitors on basal and glutamate-induced regulation of nNOS (A) and iNOS (B) mRNA expression. Bovine chromaffin cells were challenged for 24 hours with indicated concentrations of protein kinase inhibitors in the absence (-Glu) or presence (+Glu) of 2 mM glutamate. nNOS (A) and iNOS (B) mRNA expression was measured by quantitative real time PCR techniques, as described in Material and Methods. Data were expressed as a quantification of results over basal control or Glu alone. Statistic compares the effect of protein kinase inhibitors on nNOS or iNOS mRNA expression at basal levels (***) or glutamate-induced levels (°°°). (* p<0.05; ** p<0.01; ***p<0.001) (One-way ANOVA test).

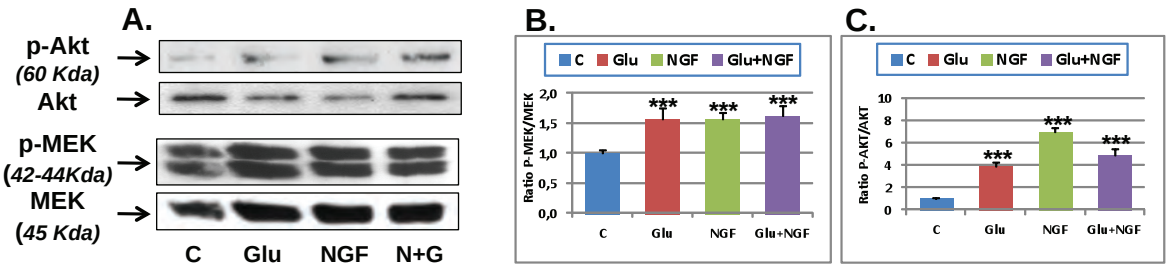


Figure 10. Participation of different signalling pathways in glutamate-induced aapoptosis.
Bovine chromaffin cells were incubated for 24h with the indicated concentrations of protein kinase inhibitors (A) Akt and MEK activation was measured by western blot. The bar graphs show the levels of activated Akt (ratios levels of pAkt-Ser-473/ total Akt) and MEK (ratios P-MEK(p44-42) Thr202/Tyr204)/total MEK) and representative blots. The data are expressed as mean ± SEM; * P =0.05; n = 3.

the NO donor SNP, which prevented translocation in a 50%.
The presence of 1 mM Thiocitrulline, which under basal conditions increase NFκB translocation at short times (30 min-1h) (Pérez-Rodríguez et al., 2009) produced a very small increase in NFκB activation in the presence of glutamate, similar or smaller than that produced by this nNOS inhibitor by itself (Figure 8A). In addition, the stimulation of cells with DETA-NO, a NO donor, was found to increase NFκB translocation at low concentrations (1 µM), but not at high concentrations (100 µM) (Figure 8B). At this high concentration, DETA-NO increases NO levels until 30-40 nmol/106 cells, a level very similar to that induced by 1 mM SNP.

5.2 A number of signalling pathways could mediate glutamate-induced nNOS activation and apoptosis in chromaffin cells

In order to know which signalling pathways could be involved in glutamate-induced NOS activation, nNOS mRNA expression experiments were performed in the presence of specific inhibitors of different signalling protein kinases, such as PKA, PKG, PKC and MAPK. Results from Figure 9A show that while basal nNOS mRNA expression was only inhibited by p38MAPK inhibitor SB203580 and MEK inhibitor PD8059, glutamate-induced nNOS expression was inhibited by 5 and 12 µM KT5823 (PKG, PKA and PKC inhibitor), SB203580 (p38MAPK inhibitor), JIP1 (JNK inhibitor), and U0126 and PD8059 (MEK inhibitors). KT 5823 inhibits PKs in function of its concentration. 1µM KT5823 is meant to inhibit specifically PKG; as the only inhibition is observed at concentrations higher than 5µM, PKG does not seem to be involved on nNOS mRNA regulation. These inhibitory effects were not found in iNOS expression (Figure 9B). Therefore, these data suggest the participation of PKA, PKC and MAPKs pathways in glutamate-mediated nNOS activation in chromaffin cells. To confirm the possible involvement of ERKs in glutamate-induced apoptosis, the ability of glutamate to induce the MEK phosphorylation was tested. Results from Figure 10A demonstrate that glutamate is able to increase the phosphorylation of MEK by about 1.7 times basal levels, which is similar to that induced by NGF. Moreover, because LY294002, a specific PI3K inhibitor, increased glutamate-induced apoptosis (Figure 10A) the ability of glutamate to increase Akt phosphorylation was also assayed. Results in Figure 10C demonstrate that 1 mM glutamate is also able to increase AKT phosphorylation in a great extension, although this effect was lower than that induced by NGF, this action being reduced in the presence of glutamate.

DISCUSSION

Nitric oxide (NO) is a highly reactive radical that plays an important role in the regulation of vascular tone, neuronal transmission and modulation of immunological and inflammatory reactions (Bredt and Snyder 1990; Moncada et al. 1991; Hierholzer et al. 1998). It has also been demonstrated that NO is involved in crucial physiological events such as the regulation of neurotransmitter release, and in pathological events underlying neurotoxicity and neurodegeneration (Dawson and Dawson, 1996, Prast and Philippu 2001, Boje 2004).
Glutamate is the major excitatory neurotransmitter in the mammalian nervous system and it plays an essential role in learning and memory (Bliss and Collingridge 1993; Reis et al. 2009). It is present in high concentrations in the adult nervous system and is released from nerve terminals after calcium signals, contributing to excitatory neurotransmission, counting for actions in the development and plasticity of synapses, among others. Excessive activation of GluR, however, is implicated in many neurological disorders. Intense stimulation of these receptors may lead to necrosis, but a mild or chronic overstimulation results in apoptotic death (Nakamura et al. 2007).

1. Chromaffin cells express iGluR and mGluR functionally active on CA secretion which is mediated by NO.

There are two families of GluR in the nervous system: the ligand-gated ionotropic receptors, NMDA, AMPA and KA, and the G-protein coupled metabotropic receptors. Among all these receptors, the NMDAR channels are very permeable to calcium (Nakamura et al. 2007). Excessive activation of NMDA receptors

contributes to cell death, by production of ROS and RNS free radicals (Dawson et al. 1991).
Our research group has worked for a long time on the secretory properties of bovine chromaffin cells, otherwise a very useful model of neurosecretory cells (Garcia 2002). We demonstrated that NO modulates CA secretion evoked by nicotine in a dose-dependent manner, through a mechanisms mediated by cGMP (Oset-Gasque et al. 1994). Also, NO donors induce calcium mobilisation from internal stores, but do not affect catecholamine secretion in resting conditions (Vicente et al. 2005). We also showed that glutamate might be involved in the regulation of CA secretion by interacting specifically with different glutamate receptor subtypes: NMDA, AMPA, KA and t-ACDP (González et al., 1998; Arce et al., 2004).
In this work, we further support those results, and demonstrate that the main ionotropic glutamate receptors (NMDA, AMPA and KA) and the specific class I excitatory glutamate receptors (mGluR1 and 5), are expressed in primary cultures of bovine chromaffin cells. Furthermore, glutamate treatment of chromaffin cells upregulated the mRNA expression of NMDA1, NMDA2A, mGluR1 and mGluR5.
Long term potentiation (LTP) is a form of memory, dependent on NMDA receptors. Most NMDA receptors are an assembly of two NMDA1 and two NMDA2A or NMDA2b subunits, showing these subunits different regulation (Dingemans et al. 2007). Also, it is known that NMDA-dependent LTP is induced by strong depolarization and a large increase in intracellular calcium levels (Lynch 2004), conditions elicited by exposure to high glutamate levels such as the ones shown in this paper. Taken all together, these results suggest that bovine chromaffin cells could also make a good model for the study of memory processes on neuronal-type cells.
In order to gain further insight into the secretory role of glutamate on chromaffin cells, we investigated the role of glutamate and glutamate agonists on CA secretion. All glutamate and glutamate agonists were able to induce CA secretion in a dose dependent manner, the order of potency being AMPA>NMDA=t-ACPD>Glu>KA. However, although these results were lower than the ones observed with nicotine, the role of glutamate as a neurosecretory agent in chromaffin cells of the adrenal medulla seems evident.
NMDAR have been shown to form part of a specialized structure beneath the postsynaptic membrane, known as the postsynaptic density (PSD). nNOS is also located in the PSD, indirectly bound to NMDA2 by the protein PSD-95. This interaction is pretty interesting, since constitutive NOS isoforms depend on calcium concentrations to become functional, and glutamate induces an inward current of calcium cations that might account for the activation needs of cNOS. For many years now it is well known that increased levels of neuronal calcium, and the binding of calcium-calmodulin, trigger the activation of nNOS and subsequent production of NO from L-arginine (Dawson et al. 1991; Abu-Soud et al. 1994). Therefore, it seems plausible that NO could be involved in many of the physiological and pathological roles of glutamate, establishing a relationship between both neurotransmitters. By using the specific NOS inhibitor thiocitrulline, we demonstrate in this paper that NOS/NO/cGMP is involved in the mechanisms whereby glutamate and glutamate agonists stimulate CA-secretion in chromaffin cells.

2. Glutamate induces apoptosis in chromaffin cells which is mediated by NO resulting from nNOS activation.

Interactions between NO and glutamate are not only related to CA secretion. Glutamate also plays an important role in excitotoxicity (Kostandy 2012). A well known relationship between glutamate, NO and apoptosis have been studied for years in many cell types (Brown 2010), but not in chromaffin cells. In previous studies, our group postulated that exogenous NO generated by NO donors plays an apoptotic role in chromaffin cells (Vicente et al, 2006). NO donors induce apoptotic cell death mediated by NO and peroxynitrites, conclusions also supported in the literature (Nomura 2004).
We proved in this paper that glutamate induces an increase in nitrite production, specific of different glutamate receptor agonists. We also assessed that glutamate and glutamate agonists induce apoptosis in a dose-dependent and synergic manner, results also supported in the literature in the well known pheochromocitoma cell line PC12 (Ma et al. 2012). Treatment with specific glutamate antagonist showed the participation of ionotropic AMPA and NMDA, and metabotropic glutamate receptors on apoptosis. On our primary cultures of bovine chromaffin cells, the glutamate antagonists NBQX, D-AP5 and MCPG were able to counteract the apoptotic effects of glutamate. Similar results are also observed in the literature, and promoted the use of glutamate antagonists to block excitotoxicity in hypoxic-ischemic patients (Won et al. 2002).
All in all, these results pointed at a relationship between glutamate and NO on apoptosis of chromaffin cells. Nevertheless, most cell types produce NO via different NOS isoforms, providing the cell with a basal and endogenous source of NO. NOS isoforms present a tissue-specific localization: neurons mainly express nNOS, but also iNOS in pathological conditions such as trauma, inflammation and ischemia. We found that chromaffin cells express nNOS at a basal level and iNOS when stimulated with cytokines (Vicente et al., 2006; Pérez-Rodríguez et al., 2009), responding these enzymes to a different regulation.
Cytokines and glutamate seem to regulate either activity or expression of the different NOS isoforms (Alderton et al. 2001; Bolaños et al. 1997; Bolaños et al., 2004). Our studies with cytokines and NOS inhibitors (Pérez-Rodríguez et al., 2009) made us conclude that these stimuli are enough to induce apoptotic death in bovine chromaffin cells due, at least in part, to NO and peroxynitrites originated from any of the NOS isoforms.
In this paper, we wondered whether glutamate

could also regulate NOS expression. Therefore, we studied the effect of endogenously generated NO (basal and after stimulation with glutamate) on both neurosecretion and apoptosis, and examined the possible involvement of nNOS and iNOS isoforms and transcripcional factor NFκB in glutamate-induced apoptosis.

Cells were exposed to high levels of glutamate in the presence and absence of NOS inhibitors, and nitrite production and apoptosis measured. It proved evident that glutamate-induced apoptosis and nitrite production was reverted by specific nNOS inhibitors in a dose-dependent manner, but no effect was seen with iNOS inhibitors. Also, nNOS inhibitors, but not iNOS, were able to induce apoptosis on basal conditions. These results point to NO from nNOS as a cytoprotector under basal conditions, but an excytotoxic in glutamate-stimulated conditions, whereas iNOS does not seem to be significantly involved in glutamate-mediated apoptosis nor nitrite production. It also seems evident that glutamate and cytokines exert NO-mediated apoptosis through different pathways (Pérez-Rodríguez et al. 2009).

We also observed that cytokines induced apoptosis on chromaffin cells, increasing iNOS, but not nNOS, mRNA levels (Pérez-Rodríguez et al. 2009). To continue with the parallelisms, we investigated whether glutamate could also regulate mRNA of any of these isoforms. Opposite to cytokine results, we observed that glutamate and glutamate agonists upregulated nNOS mRNA levels, and had no effect on iNOS levels. Also, NOS inhibitors differently reverted this upregulation on nNOS, and had no effect on iNOS. Same results were observed at protein level, and also NOS inhibitors were able to increase nNOS expression on their own. From these results, it seems plausible that glutamate-upregulated nNOS mRNA and protein is maybe mediated by a mechanism involving nNOS generated NO levels. In fact, previous work from our group demonstrated that NO is able to induce glutamate release from chromaffin cells (Romero et al. 2003; unpublished results from our group).

Taken all together, and opposite to what we observed with cytokine stimulation, these results confirm that nNOS is the isoform mainly involved in the synthesis of NO that mediates the apoptotic effect of glutamate in chromaffin cells.

3. PKA, PKC, PKG and ERKs pathways activation, but not NFκB, are involved in the signalling mechanisms of glutamate-induced apoptosis and nNOS expression in chromaffin cells

In a different approximation to the role of NOS in apoptosis, we focused on the ztranscriptional factor NFκB stays inactive in the citosol, but under some conditions, it translocates into the nucleus and activates the synthesis of the iNOS (Aktan 2004). We stated that under cytokine stimulation, the apoptotic effect observed in chromaffin cells, was mediated by NFκB activation and induction of iNOS (Pérez-Rodríguez et al, 2009). By stimulating chromaffin cells with glutamate, our results here suggest that glutamate does not activate NFκB translocation or iNOS expression, being its apoptotic effect mediated exclusively by nNOS activation. Moreover, exogenous NO coming from high concentrations of NO donors would involve inhibition of NFκB activation, decreasing its appearance in the nucleus at different times (Pérez-Rodríguez et al. 2007). On the other hand, in basal conditions, thiocitruline and N-PLA basally induced NFκB activation showing that nNOS inhibition in basal conditions was enough to activate NFκB , and thus the basal NO levels generated by nNOS block NFκB activation (Pérez-Rodríguez et al., 2009)..This paradox could be explained by the NO concentrations produced by nNOS. Basal nNOS produces low NO concentrations, inhibiting NFκB activation. However, after pathological glutamate-induced activation of nNOS, NO may produce peroxynitrites, and S-nitrosylation of the redox sensitive NFκB . S-nitrosylation of the p50 subunit of the NFκB inhibits its DNA binding activity; Also, S-nitrosylation of IκB prevents its phosphorylation and dissociation from NFκB , preventing its translocation to the nucleus (Pannu and Singh 2006).

In relation with other possible signalling mechanisms involved in glutamate-induced apoptosis in chromaffin cells, it is known that NO regulates apoptosis by pathways involving multiple protein kinases. The action and importance of these kinases depends on the cell type examined. It has been observed that cells cultured with different NO donors proved different actions on PKA, PKC and MAPK signalling; both pro-and anti-apoptotic depending on the cell type (Kim et al. 2001). MAPKs are expressed in the nervous system, and alterations in MAPK expression and/or activation in post-ischemic brain tissues can affect the outcome of ischemic brain injury in animal models. MAPKs are divided in three families: extracellular signal regulated kinases (ERKs), c-Jun-N-terminal kinases (JNKs), and p38 MAPKs, each of which play crucial roles in signal transduction and regulate cell death and survival. In our cell system, many MAPK pathways seem to be simultaneously activated by glutamate (data not shown), in special ERKs activation pathway. This would also explain why sometimes pro- or anti-apoptotic effects differ from others described in the literature (Valladares et al. 2000). This process of apoptosis is not so dramatic, because antiapoptotic pathways attenuate those proapoptotic pathways that are activated simultaneously. In fact our results shown that in chromaffin cells glutamate seem to stimulate the prosurvival pathway of PI3K/AKT, and modulate its activation by NGF, at the same time that it triggers the activation of apoptotic pathways like ERKs. NMDA receptor-mediated neurotoxicity involving activation of the MEK/ERK pathway and some apoptotic mechanisms has been supported by the literature (Kurakowa et al., 2011).

Our results seem to suggest the participation of PKG, PKA, PKC and MAPKs pathways in glutamate-mediated nNOS activation in chromaffin cells.

PKA-mediated phosphorylation of nuclear and cytoplasmic substrates is critical for multiple cell functions. PKA is activated by increased intracellular cAMP concentrations (Karaçay et al. 2007). In many systems, activation of PKA by extracellular ligands or cAMP inhibits apoptosis, for instance by phosphorylating and inactivating the pro-apoptotic Bad (Feliciello et al. 2005). NOS protein regulation can be achieved by phosphorylation of some residues, therefore showing a role for kinase proteins. However, it has been shown by us and others, that protein phosforylation is not the only way for kinase proteins to regulate NOS, since many pathways mediated by these proteins may end up in NOS RNA regulation (Pérez-Rodríguez et al. 2007). We show in this paper that iNOS was not affected at RNA levels by any of the kinases tested, however nNOS was. One of the clearest results was obtained with PKA activators (data not shown) and inhibitors. These interactions are mediated by CRE (cAMP response element). The nNOS gene promoter contains two cAMP response elements. Therefore, nNOS might be a downstream target of the cAMP signalling pathway, and activation of the pathway induces nNOS gene expression (Karaçay et al. 2007), as also observed here.

CONCLUSIONS

In short, at the view of our results and the evidences on the literature about endogenous NO and apoptosis, we propose a model to explain the effects of NO, both endogenous and cytokines/glutamate stimulated on bovine chromaffin cells (Figure 11).

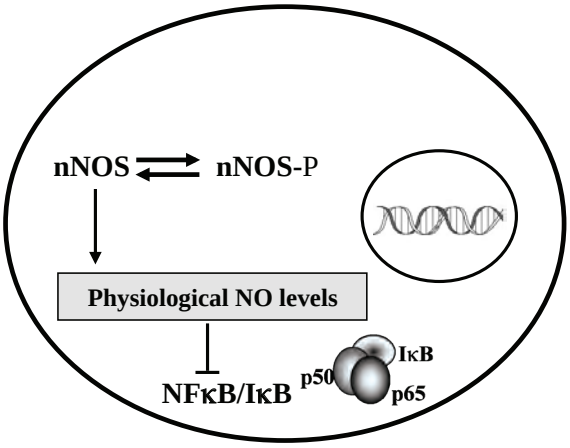
1- In basal conditions, chromaffin cells express only nNOS, but not iNOS, having nNOS inhibitors an apoptotic effect. Therefore, in basal conditions nNOS is anti-apoptotic and cytoprotective, promoting cell survival. NO physiological concentrations of NO produced by nNOS would be enough to stop NFkB activation, since it has been observed that nNOS inhibitors promote its activation (Figure 11a)

2- Glutamate, possibly through NMDA- and non NMDA-type iGluRS and mGluRs, would activate nNOS, producing NO enough to stimulate apoptosis and inhibit NFkB activation, which could not react as a survival factor. The presence of nNOS inhibitors would counteract these glutamate-induced NO levels, improving cell survival, but yet not activating NFkB since NO remains in a range of concentrations enough to block it. (Figure 11b)

3- Our results agree with the hypothesis of a NO regulation of NFκB activation in a dose-dependent manner, this effect looking like a double Gauss bell curve. Activation of NFκB is blocked in basal conditions by physiological concentrations of NO constitutively synthesized by nNOS. However, NO concentrations much lower than basal levels (may be obtained through enzyme inhibitors or enzyme phosphorylation) would block NFκB inhibition, contributing to iNOS activation and apoptosis. In the same way, very high levels of glutamate-generated NO, would block redox sensitive NFκB activation, thereby induced peroxynitrite formation and S-nitrosylation, restraining induced apoptosis.

4- NFkB would thereby act as a survival or death factor, depending on the pathways involved and the cell conditions. This double role would mean that small amounts of NFκB could permit cell death, whereas a great activation would stop it. Activation/inhibition much depends on the time curse of cell and molecular events.

A) Basal conditions



B) Glutamate

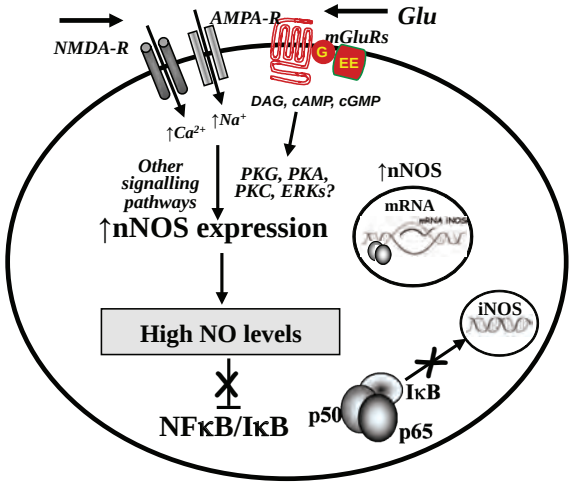


Figure 11. Glutamate induction of nNOS expression and inhibition of NFkB activation in bovine chromaffin cells.
Under basal conditions (A) chromaffin cells express nNOS, which produces physiological and cytoprotective amounts of NO, thereby blocking NFκB translocation to the nucleus. Glutamate (B), by promoting Ca2+ increase and PKA/PKC/PKG activation, mediated by both iGluR and mGluRs activation, could stimulate nNOS expression, leading to high and toxic concentrations of NO, involved in apoptosis, which blocks NFκB activation and lead to an inhibition of iNOS expression.

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VII. Discusión

En 1998, los doctores Ignarro, Murad y Furchgott recibieron el premio Nobel de Medicina por el descubrimiento del "Óxido nítrico como molécula de señalización en el sistema cardiovascular". Desde entonces, esta molécula ha despertado un gran interés en el mundo científico, siendo ampliamente estudiada y descrita en la literatura. El NO es un mensajero celular, que juega un papel muy importante en diversos mecanismos, como la regulación del tono vascular, la neurotransmisión y la modulación de procesos inmunológicos e inflamatorios (Madhusoodanan & Murad 2007). Todas las células nucleadas de mamíferos en las que se ha estudiado son capaces de sintetizar NO de manera endógena, a partir del aminoácido L-arginina, en un proceso catalizado por la familia de proteínas óxido nítrico sintasas (NOS), (Bogdan 2001).

La muerte neuronal de tipo apoptótico ha sido ampliamente descrita en los procesos de neurotoxicidad y neurodegeneración (Lirk et al. 2002). En el sistema nervioso se puede inducir la producción de grandes cantidades de NO en respuesta a citoquinas (Liu et al. 2002), o a una excesiva liberación de glutamato (Nakamura et al. 2007). En estas condiciones, el NO liberado está relacionado con la neurotoxicidad y la neurodegeración. Pero el papel del NO en la regulación de la apoptosis es más complejo, pues puede actuar tanto como agente pro-apoptótico como anti-apoptótico (Boyd & Cadenas 2002). Por ello, el estudio de la regulación del NO en estos procesos se ha convertido en una de las actuales metas de la investigación mundial.

Las citoquinas son unas importantes moléculas señalizadoras en procesos inmunes e inflamatorios (Bunn et al. 2012). Por su parte, el glutamato es el principal neurotransmisor excitatorio en el sistema nervioso, implicado en la formación de memoria y la plasticidad neuronal, pero también en los peligrosos procesos de excitotoxicidad (Bliss & Collingridge 1993; Kostandy 2012), término que se acuñó prácticamente para poder describir la toxicidad de la hiperexcitación por glutamato. Además, existe una interesantísima relación entre el glutamato y el NO, tanto a nivel físico, como fisiológico y patofisiológico. Para nuestro estudio escogimos el modelo de células cromafines de origen bovino como modelo de células neurosecretoras, modelo que se empezó a utilizar en los años 60 del siglo pasado (Pearse 1966), y que por sus excelentes cualidades sigue en vigencia en la actualidad (Garcia 2002). Tanto es así, que en 1991 los Doctores Neher y Sakmann recibieron el premio Nobel de Fisiología por sus estudios de patch-clamp, realizados con células cromafines. Además, experimentos previos en nuestro grupo mostraron que este tipo de células presentan receptores específi-

cos de glutamato y citoquinas (González et al. 1998; Vicente et al. 2002), abriendo una puerta al estudio el papel del NO en la apoptosis en este modelo celular.

Se podría decir que la muerte celular de tipo apoptótico es una muerte altruista. El último acto de generosidad de una célula para con sus vecinas (Savill et al. 1993). Las células que mueren por apoptosis muestran marcadores en su membrana que facilitan que sean rápidamente secuestradas por fagocitos, antes de que puedan liberar su contenido al medio extracelular y causen una reacción inflamatoria. Esto es de vital importancia en el sistema nervioso. La concentración de glutamato intracelular en el cerebro es aproximadamente 3-10 mM en el citosol, 100 mM en las vesículas sinápticas, mientras que la excitotoxicidad cursada por glutamato se produce a concentraciones de tan sólo 10-20 µM. La lisis necrótica liberaría todo este contenido intracelular de manera descontrolada, causando un importantísimo daño celular (Leist & Nicotera 1998). No es de extrañar, entonces, que las células del sistema nervioso regulen de manera tan exhaustiva su muerte celular.

1. ESTUDIOS DE NO EXÓGENO

En estudios llevados a cabo por nuestro grupo de investigación, se caracterizó la acción pro-apoptótica de los donadores de NO (Vicente et al. 2006). En este estudio se observaba que los donadores de NO producían una muerte de tipo apoptótico, mediada tanto por NO como por peroxinitritos, éstos últimos también producían muerte necrótica, conclusiones apoyadas por la literatura (Nomura 2004), que concurría con la activación de caspasas.

Continuando con esta línea investigadora de nuestro grupo, y en una primera etapa del proceso, caracterizamos la ruta mitocondrial apoptótica inducida por donadores exógenos de NO, destacando el transcurso temporal de los acontecimientos.

Se ha descrito en la literatura que el NO puede iniciar vías de señalización apoptóticas mediante la formación de peroxinitritos, la modulación de señales oxidativas y la liberación de zinc de almacenes intracelulares. La liberación de zinc induce una disminución transitoria en el potencial de membrana mitocondrial y la subsecuente liberación de citocromo c (Bossy-Wetzel et al. 2004). Al mismo tiempo, se produce la activación de las caspasas 3 y 7, que producen un aumento de la transcripción de p53 y BAX (Wang et al. 2008). Una característica de los donadores de NO es que difieren en su capacidad temporal de liberar NO, de esta manera, en función de los donadores de NO que estemos utilizando, se producirá un diferente patrón en la liberación de NO y, por tanto, en las vías implicadas.

1.1 Los donadores de NO inducen la despolarización del potencial de membrana mitocondrial, y esto precede a un aumento de la apoptosis

Al incubar nuestras células con los donadores de NO SNP y SNAP, observamos que, como se ha demostrado también en otros tipos celulares, se inducía un descenso progresivo en el potencial de membrana mitocondrial, a la vez que se producía un aumento en la apoptosis y de la activación específica de caspasa-3. Esta permeabilización de la membrana se puede conseguir también con otros estímulos, como ROS, calcio (Kim et al. 2001), o glutamato. La despolarización del potencial de membrana mitocondrial está relacionada con la permeabilidad de la membrana inteARN de la mitocondria. Esta permeabilidad no debe entenderse sólo como el paso de iones a su través, sino también como la formación física de pequeños poros, a través de los cuales se escapan proteínas, como el citocromo c (Kim et al. 2000). Todo ello contribuye a la generación de ROS, aumentando el daño a la mitocondria. Este proceso podría seguir retroalimentándose eteARNmente pero, eventualmente, se inicia la activación de caspasas, concurriendo en apoptosis (Leist & Nicotera 1998).

1.2 Los eventos implicados en la apoptosis inducida por los donadores de NO muestran un claro transcurso temporal

Según lo expuesto anteriormente, cabría esperar que en el transcurso temporal de la apoptosis inducida por NO en las células cromafines, se observara una salida del citocromo c, posterior a la despolarización de la membrana. Efectivamente, con experimentos de western blot demostramos la aparición progresiva de la proteína citocromo c en el citosol, y su disminución en la mitocondria, ambos procesos posteriores a la despolarización. Además, en nuestro modelo, la liberación de citocromo c precedía a la activación de caspasa 3 y a la presencia de células hipodiploides. Esto concuerda con resultados obtenidos en modelos de neuronas corticales y motoneuronas (Figueroa et al., 2006).

En nuestros estudios, resaltamos la temporalidad en los eventos que median la apoptosis inducida por donadores de NO. En primer lugar observamos la despolarización del potencial de membrana mitocondrial, posteriormente la liberación de citocromo c al citosol, y más tarde la activación de caspasas, indicando que la vía implicada en la apoptosis inducida por donadores de NO en las células cromafines bovinas, la activación de caspasa 3 se produce por la vía intrínseca o mitocondrial.

También demostramos que los donadores de NO alteran la expresión de los miembros pro-apoptóticos y anti-apoptóticos de la familia Bcl-2. De este modo, disminuyen la expresión de los miembros antiapoptóticos de la familia Bcl-2, Bcl-2 y Bcl-xL, y aumentan la expresión de de los miembros pro-apoptóticos, Bax y Bcl-Xs. La

importancia de estos datos no radica en el aumento o la disminución de la expresión de una proteína en concreto, sino más bien de la ratio Bax/Bcl-2, como se propone en la literatura (Harris and Thomson 2000; Chae et al. 2004).

Estos factores inducen la liberación de citocromo c al citosol, y por lo tanto se sitúan antes en la cascada de señales apoptóticas. Como los menores niveles de Bcl-2 se observan más tarde que el aumento de los niveles de Bax, ambos mecanismos parecen ir por vías de señalización diferentes.

1.3 Los donadores de NO inducen la fosforilación de Bad en el residuo serina 136

Bad es un miembro pro-apoptótico de la familia Bcl-2, que promueve la apoptosis formando heterodímeros con el factor anti-apoptótico Bcl-2, evitando que se una a Bax. La fosforilación de Bad, un mecanismo muy común de regulación proteica, promueve la liberación de Bcl-2, conduciendo a un efecto antiapoptótico (Datta et al. 1997). Nuestros datos confirman los mostrados en la literatura, de manera que la acción de los donadores de NO estimularía la fosforilación de Bad, promoviendo un efecto de supervivencia celular ante la toxicidad del estímulo, y aumentando el umbral al que la mitocondria liberaría el citocromo c.

1.4 Los donadores de NO aumentan la expresión de p53

La muerte inducida por NO puede seguir un mecanismo dependiente o independiente de p53 (Messmer and Brune, 1996; Brune et al 1998; Kibbe et al. 2002). Estos autores han descrito que el NO produce la acumulación de p53, que a su vez actúa como activador de la transcripción de Bax, e inhibidor de Bcl-2.

En nuestros estudios, confirmando lo descrito en la literatura, observamos que p53 aumenta su expresión con los donadores de NO, en una fase temprana de la apoptosis, y podría ser responsable de la posterior inducción en la expresión de Bax.

Con estos resultados, demostramos el papel de los mediadores mitocondriales en la apoptosis inducida por donadores de NO, y su transcurso temporal, en las células cromafines bovinas.

2. ESTUDIOS DE NO ENDÓGENO

Hasta este punto, utilizamos un modelo experimental con un aporte exógeno de NO mediado por donadores de NO. Esta aproximación es muy útil, pues prácticamente todos los tipos celulares están expuestos a la presencia de NO. Por otra parte, el NO se genera de manera endógena (Bogdan 2001), en una reacción de oxidación catalizada por la enzima óxido nítrico sintasa (NOS). Citoquinas (Liu et al. 2002), glutamato (Nakamura et al. 2007) y agonistas de glutamato parecen regular bien la actividad, bien la expresión, de las distintas isoformas (Bolaños et al. 1997; Alderton et al. 2001). Trabajos previos realizados en nuestro grupo de investigación con citoquinas y glutamato y sus agonistas, así como con inhibidores de NOS, nos han llevado a la conclusión de que las células cromafines presentan receptores específicos para ambos, y que estos estímulos son suficientes para inducir una muerte apoptótica en las células cromafines bovinas, debida, al menos en parte, a NO y peroxinitritos, de origen mediado por alguna de las isoformas de NOS (Vicente et al. 2002; Vicente et al. 2006), como también se observa en la literatura (Calabrese et al. 2007).

2.1 Las células cromafines expresan iGluR y mGluR, funcionalmente activos, que secretan catecolaminas en un proceso mediado por NO.

Tanto el NO como el glutamato son neurotransmisores del sistema nervioso de mamíferos. El glutamato es el principal neurotransmisor excitatorio en el sistema nervioso de mamíferos. Además de estar implicados en muchas funciones fisiológicas, en condiciones patológicas los dos neurotransmisores están relacionados con procesos de neurotoxicidad y neurodegeneración, donde el NO puede actuar como mensajero retrógrado sobre el glutamato (Jacintho & Kovacic 2003).

En el sistema nervioso hay dos familias de GluR: los receptores ionotrópicos, iGluR (NMDA, AMPA y KA), y los receptores acoplados a proteínas G, mGluR (1-8). De todos ellos, el receptor NMDA es el más permeable a calcio (Nakamura et al. 2007). Una activación excesiva de glutamato está asociada con procesos de muerte celular y formación de las especies reactivas de oxígeno ROS y RNS (Dawson et al. 1991). Además, este receptor posee una característica fundamental para nuestros estudios. El NMDAR forma una estructura especializada en las membranas postsinápticas (PSD), donde, de manera indirecta, se encuentra unida la isoforma nNOS, que se activa en presencia de calcio (Abu-Soud et al. 1994).

Las células cromafines tienen una gran importancia fisiológica en la secreción de catecolaminas mediada por glutamato (González et al. 1998; Arce et al. 2004). En estas células, el NO es capaz de inducir la liberación de calcio desde vesículas inteARNs, pero no puede por sí mismo regular su secreción (Vicente et al. 2005).

Por todo ello, nos planteamos la conveniencia de caracterizar el tipo de receptores glutamatérgicos presentes en las células cromafines. La presencia de el NMDAR podría ser indicativo de concurrencia de nNOS, y de este modo el NO podría tener un efecto neuromodulador en la secreción de catecolaminas inducida por glutamato, además de en otros procesos fisiológicos y patológicos.

De este modo, demostramos que las células cromafines bovinas expresan los principales iGluR, NMDA, AMPA y KA, y los mGluR específicos de clase I mGluR1 y 5. Además, demostramos que la estimulación con glutamato sobreexpresaba las subunidades NMDA1, NMDA2A y mGluR1 y mGluR5. Estos datos además abren una puerta al uso de células cromafines bovinas como modelo de neuroplasticidad, ya que los procesos de formación de memoria dependen de la regulación de la expresión de NMDA1 y NMDA2, en procesos regulados por glutamato (Lynch 2004; Dingemans et al. 2007).

2.2 Las células cromafines bovinas muestran actividad enzimática NOS dependiente e independiente de calcio, que se inhibe de manera específica con inhibidores nNOS e iNOS, respectivamente.

Aunque como hemos dicho con anterioridad casi todos los tipos celulares son capaces de producir NO, la NOS presenta tres isoformas, con una distribución específica de tejido. En el Sistema Nervioso se encuentran las tres isoformas de la NOS (Esplugues 2002), pero la principal isoforma expresada en las neuronas es la nNOS (Förstermann et al. 1998).

Una de las principales diferencias en estas isoformas, es la regulación de su actividad dependiente de calcio. Todas las isoformas presentan una zona de unión a calmodulina. La calmodulina funciona como un activador alostérico de las NOS (Abu-Soud et al. 1994). Se dice que las isoformas constitutivas son dependientes de calmodulina porque nNOS y eNOS contienen un inserto que desestabiliza la unión de calmodulina a bajas concentraciones de Ca^{2+} e impidiendo la transferencia de electrones. En este caso, las enzimas son catalíticamente inactivas (Nishida & de Montellano 1999; 2001). Este inserto, sin embargo, no está presente en la iNOS (Kone et al. 2003).

En este trabajo, y de acuerdo con los datos observados en la literatura, demostramos la presencia en las células cromafines bovinas de enzimas con actividad NOS, de manera dependiente e independiente de calcio. En nuestro modelo, de manera constitutiva, las células cromafines tienen actividad enzimática NOS principalmente dependiente de calcio, pero también y de manera minoritaria, con independencia de calcio. Tras la estimulación con citoquinas (IFNγ) aumenta la actividad enzimática de la NOS independiente de calcio, pero no de la dependiente de calcio. La ac-

tividad enzimática de la NOS dependiente de calcio se inhibe por los inhibidores específicos de la nNOS N-PLA y tiocitrulina, pero no por el inhibidor de la iNOS W-1400, lo cual indica que la NOS dependiente de calcio en nuestro modelo celular es la nNOS. La actividad enzimática de la NOS independiente de calcio se inhibe por el inhibidor específico de la iNOS W-1400, pero no por los inhibidores específicos de la nNOS N-PLA y tiocitrulina; de este modo, las citoquinas pueden inducir la presencia de la isoforma independiente de calcio iNOS, pero no ejercen efecto sobre la nNOS.

2. 3 Las células cromafines bovinas expresan las isoformas nNOS e iNOS, pero no eNOS, que se regulan de manera diferente por citoquinas, glucocorticoides y glutamato y sus agonistas.

En la literatura se ha descrito que las citoquinas (Turquier et al. 2002, Vicente et al. 2006) y el glutamato (González et al. 1998; Arce et al. 2004), como también hemos descrito anteriormente, son capaces de inducir la generación de NO, efectos que podrían estar mediados por glucocorticoides como la dexametasona (Korhonen et al. 2002; Golde et al. 2003; Shinoda et al. 2003). Por ello, nos propusimos estudiar el efecto de estos compuestos en la expresión de las NOS en las células cromafines bovinas, tanto a nivel de ARN como de proteína.

Observamos que las citoquinas (LPS, IFN-γ) y TNF-α no regulan la nNOS, ni a nivel de mARN, ni a nivel de proteína, pero inducen el aumento del mARN de la iNOS, y su expresión proteica, de manera dosis-dependiente. Los glucocorticoides (dexametasona) inducen el aumento del mARN de la nNOS, y su expresión proteica, de manera dosis dependiente, pero no regulan la iNOS ni a nivel de mARN, ni a nivel de proteína. Por último, el glutamato, y los agonistas glutamatérgicos (NMDA, AMPA y t-ACPD), regulan la nNOS. El glutamato aumenta los niveles de mARN de la nNOS. El glutamato y los agonistas de glutamato NMDA, AMPA y t-ACPD, aumentan la expresión de la proteína de la nNOS de manera dosis-dependiente. La iNOS no se ve regulada por glutamato.

2.4 La estimulación con citoquinas y glutamato induce disminución en la viabilidad celular y aumento de la apoptosis y la producción de nitritos

Como ya hemos comentado previamente en esta discusión, tanto las citoquinas como el glutamato están relacionados con el daño celular de tipo apoptótico (Bolaños et al. 1997; Alderton et al. 2001).

En nuestro modelo de células cromafines, demostramos en este trabajo que la estimulación con citoquinas produce una disminución en la viabilidad celular y aumento de la apoptosis, datos todos

ellos correlacionados con el aumento en la producción de nitritos. Los mismos efectos son observados en la estimulación con glutamato, y con sus agonistas, que fueron capaces de inducir apoptosis de manera dosis dependiente. Además, se observó una reacción sinérgica apoptótica entre receptores, y se observó que estos resultados eran específicos de receptor, al tratar a las células con inhibidores específicos.

2.5 Las isoformas nNOS e iNOS están implicadas de manera diferente en la apoptosis inducida por citoquinas y glutamato.

En la primera parte de este trabajo observábamos como los donadores de NO eran capaces de inducir la muerte celular de tipo apoptótico. Además, otros trabajos de nuestro grupo con citoquinas y glutamato y sus agonistas indicaban que estos estímulos son suficientes para inducir una muerte apoptótica en las células cromafines bovinas, debida, al menos en parte, a NO y peroxinitritos, de origen mediado por alguna de las isoformas de NOS (Vicente et al. 2002; Vicente et al. 2006; Calabrese et al. 2007).

La implicación de cada una de las isoformas en la generación de NO es muy importante, dado el papel tanto pro-apoptótico como anti-apoptótico que puede jugar el NO, y que le ha merecido apodos como "Dr. Jekyll y Mr. Hide"(Kubes 2000). Tanto la nNOS como la eNOS, cuando se expresan de manera constitutiva, producen NO en pequeñas cantidades (en el rango nM) que ejercen funciones fisiológicas y citoprotectoras. La iNOS, que se expresa principalmente tras ser inducida (aunque se ha observado su expresión constitutiva en algún tipo celular) por estímulos como las citoquinas, produce cantidades de NO en el rango µM. Cuando las NOS constitutivas se activan por largos periodos de tiempo, o cuando se activa la iNOS, se producen altas concentraciones de NO y peroxinitritos, potencialmente peligrosas (Liu et al. 1995; Kwon et al. 2003; Domenico 2004).

Por lo tanto, y para caracterizar la procedencia endógena del NO, nuestro siguiente objetivo fue determinar la implicación de estas isoformas en la apoptosis de las células cromafines bovinas. Para ello, evaluamos la viabilidad celular, la apoptosis y la producción de nitritos tanto de manera basal (datos no mostrados), como tras la inducción con citoquinas y glutamato.

2.5.1 De manera basal, la nNOS produce pequeñas cantidades de NO implicadas en la supervivencia celular

A la vista de nuestros resultados, y en concordancia con los datos observados en la literatura, observamos que, en condiciones basales, la enzima constitutiva nNOS se encuentra activa, y pro-

duce NO en bajas concentraciones (concentraciones fisiológicas) que están implicadas en la supervivencia celular. En estas mismas condiciones se observa que la isoforma iNOS no tiene ninguna influencia sobre la viabilidad celular, resultado que es lógico ya que esta isoforma no se expresa de forma constitutiva en nuestras células.

La situación cambia completamente cuando las células están sometidas a estimulación. Se observa primeramente que la estimulación producida por citoquinas y la producida por agonistas de glutamato se resuelven de manera distinta, implicando que transcurren por distintas rutas metabólicas. El tratamiento de las células tanto con citoquinas como con glutamato y sus agonistas produce apoptosis mediada por NO. Sin embargo, la ruta por la que se produce este NO parece ser muy distintas para ambos tipos de moléculas.

2.5.2 La iNOS es la principal isoforma implicada en la apoptosis inducida por citoquinas, pero no en la inducida por glutamato.

Nuestros resultados muestran que las citoquinas son capaces de inducir la expresión de la iNOS, en concordancia con lo observado en la bibliografía (Turquier et al. 2002), generando grandes cantidades de NO implicadas en la disminución de la viabilidad celular mediada por apoptosis, y estos resultados se pueden revertir con inhibidores específicos de la iNOS. La manera en que se realiza esta inducción se discutirá más adelante.

En el caso de la estimulación por glutamato, a la vista de nuestros resultados, y tras no encontrar pruebas contundentes en la bibliografía, proponemos que la iNOS no está directamente implicada en la apoptosis inducida por glutamato.

2.5.3 La nNOS es la principal isoforma implicada en la apoptosis inducida por glutamato, pero no en la inducida por citoquinas.

Lo que parece deducirse claramente de nuestros resultados con inhibidores específicos de ambas isoenzimas, es que el glutamato activa la nNOS y promueve la apoptosis, por lo que la apoptosis inducida por NO vía glutamato parece estar mediada fundamentalmente por activación de esta isoenzima. De hecho, resultados anteriores de nuestro grupo de investigación han mostrado que el glutamato y sus agonistas son capaces de activar la secreción de catecolaminas por las células cromafines, a través de la producción de NO y cGMP, siendo el NO producido mediante la activación de una NOS constitutiva, Ca^{2+}-dependiente y soluble que se ha identificado como la nNOS (Vicente et al. 2002).

2.6 El factor transcripcional NF-κB está implicado en la apoptosis inducida por donadores de NO y citoquinas, pero no en la apoptosis inducida por glutamato: papel del NO

En la literatura hay evidencias que muestran que el factor nuclear NF-κB está implicado en la regulación de la expresión de iNOS, además de muchos otros genes. En condiciones basales, NF-κB se encuentra unido a IκB en el citoplasma, pero tras ciertas condiciones, como la presencia de citoquinas, NF-κB se disocia de IκB, traslocándose al núcleo y activando la síntesis de iNOS (Aktan 2004).

De este modo, pusimos a punto experimentos con los cuales observamos que las citoquinas, que inducen apoptosis, activan NF-κB, promoviendo su translocación al núcleo, y aumentando de manera la expresión de iNOS, tanto a nivel de mARN como a nivel de proteína. Este efecto era específico, pues se inhibía con agentes bloqueantes de la translocación de NF-κB al núcleo; sin embargo, no era exclusivo, pues la inhibición de la expresión de iNOS no era en ninguno de los casos superior al 50%.

Sin embargo, cuando estimulamos las células cromafines con glutamato, y al contrario de lo observado con las citoquinas, no se observaba la translocación de NF-κB al núcleo.

2.6.1 Los donadores de NO inducen la translocación de NF-κB al núcleo, sólo a tiempos cortos

Las grandes cantidades de NO que conllevan apoptosis se pueden generar tanto con donadores de NO como con la inducción de la expresión de la iNOS. Algunos investigadores han demostrado que el SNP disminuía la unión de NF-κB (Ibe et al., 2001). Nuestros experimentos con donadores de NO, al contrario de lo descrito por Ibe, demostraron que éstos inducían la translocación de NF-κB al núcleo a tiempos cortos, y que esta translocación disminuía con el tiempo.

Sería de esperar que la inhibición de la translocación de NF-κB disminuyera la apoptosis. Por ello, evaluamos el efecto de atrapantes de NF-κB, sobre la apoptosis inducida por citoquinas. Nuestros resultados fueron, sin embargo, contrarios a nuestro planteamiento, demostrando que al impedir esta translocación se produce un incremento en la apoptosis. NF-κB está implicado en la transcripción de muchos genes (Tornatore et al. 2012). Por todo ello, proponemos que NF-κB resulta un factor de supervivencia celular y no de muerte, aunque su activación no sea capaz de prevenir la apoptosis celular inducida por citoquinas, ya que activa la expresión de moléculas como la iNOS generadora de altas concentraciones apoptóticas de NO. Todos estos procesos podrían tener una dependencia temporal.

2.6.2 Modelo de regulación de las NOS mediado por NO

¿Cómo se podrían integrar estas respuestas tan diferentes? Se ha propuesto un nuevo modelo de interacción entre nNOS e iNOS, mediado por NO, que revoluciona la manera en la que se ha entendido hasta ahora la regulación de estas isoformas y que concuerda en gran medida con nuestros resultados experimentales: La regulación de iNOS por nNOS (Colasanti & Persichini 2000; Colasanti & Suzuki 2000; Suzuki & Colasanti 2001a; Suzuki & Colasanti 2001b). En este modelo se propone que la expresión de iNOS está regulada por nNOS de la siguiente manera: La nNOS produce niveles basales de NO. El NO puede S-nitrosilar NF-κB, impidiendo su unión al ADN, y por lo tanto su actividad. Por otro lado, el NO también puede S-nitrosilar IκB, impidiendo su fosforilación y consecuente degradación (Pannu & Singh 2006). De este modo, y como mostramos con experimentos con citoquinas e inhibidores de la nNOS, el NO producido de forma constitutiva por la nNOS sería suficiente para impedir la translocación al núcleo de NF-κB, que una vez allí, activaría la expresión de la iNOS, produciendo grandes cantidades de NO, y apoptosis. Por o tra parte, las grandes concentraciones de NO generados por la excitotoxicidad inducida por glutamato, podría generar peroxinitritos, que a su vez inducirían otras reacciones de S-nitrosilación, lo que explicaría nuestros resultados experimentales.

2.6.3 Las citoquinas fosforilan nNOS en 847Ser.

En el modelo propuesto, las citoquinas inhiben la fosforilación de la nNOS, impidiendo la formación de NO y favoreciendo la translocación de NF-κB al núcleo.

La fosforilación de la nNOS regula su actividad en función del resíduo fosforilado. Se ha descrito que la fosforilación en el residuo 847Ser disminuye la actividad catalítica de la enzima en muchos tipos celulares (Nakane et al. 1991; Hayashi et al. 1999). Nuestros resultados demuestran que en las células cromafines bovinas, las citoquinas inducen la fosforilación de la nNOS en este residuo, lo que observamos en paralelo con el tiempo de translocación de NF-κB al núcleo, corroborando la hipótesis planteada.

2.6.4 La fosforilación de nNOS está mediada por PKA, PKG y MEK

Por otra parte, y para continuar estudiando la fosforilación de la nNOS, estimulamos las células con citoquinas en presencia de inhibidores específicos de las proteínas quinasas. De este modo, observamos que la fosforilación de la nNOS estaba mediada por las vías de señalización de la PKA, PKG y MEK, en concordancia con datos observados en la literatura (Bredt et al. 1992).

2.7 La vía JAK/STAT está implicada en la inducción de iNOS por citoquinas

Como hemos demostrado anteriormente, NF-κB no es el único factor implicado en la expresión de la iNOS inducida por citoquinas. Datos en la literatura muestran que el promotor de la iNOS humana contiene también secuencias para otros factores de trascripción, como STAT (Spitsin et al. 1996; Linn et al. 1997). Observamos que la estimulación con citoquinas aumentaba la fosforilación de STAT-3, máxima al mismo tiempo que la fosforilación producida en nNOS. Además, demostramos que la fosforilación de STAT-3 promueve la expresión de la iNOS inducida por citoquinas, de manera específica y mediada por la vía de las JAK quinasas, en concordancia con lo observado por otros autores (Ziesché et al. 2007)

VIII. Conclusiones

El presente estudio sobre el papel del NO y de las NOS en la apoptosis de las células cromafines de la médula suprarrenal bovina, ha dado lugar a las siguientes conclusiones:

1. En las células cromafines bovinas se expresan las isoformas nNOS e iNOS, que muestran una diferente dependencia de calcio y diferente regulación por glucocorticoides, citoquinas y glutamato.

2. En las células cromafines bovinas se expresan los principales iGluR, NMDA, KA y AMPA, y los mGluR específicos de clase I, mGluR1 y mGluR5. La estimulación con glutamato aumenta la expresión del mARN de NMDA1, NMDA2, mGluR1 y mGluR5.

3. El glutamato y los agonistas de sus principales receptores iGluR y mGluR incrementan la secreción de catecolaminas mediante un proceso mediado por NO y GMPc.

4. Las citoquinas y el glutamato disminuyen la viabilidad celular, aumentan la apoptosis y la producción de NO, mediada por la activación de diferentes isoformas de la NOS, iNOS y nNOS, respectivamente.

5. En condiciones basales, las células cromafines bovinas expresan sólo nNOS. Las concentraciones fisiológicas de NO producidas por estas enzimas serían suficientes para impedir la activación de NF-κB, dado que los inhibidores de la nNOS promueven su activación. De este modo, la nNOS jugaría un papel anti-apoptótico y citoprotector.

6. Las citoquinas, probablemente por activación de la actividad tirosina quinasa de JAK, y de la vía JAK/STAT-3, inducen la fosforilación de nNOS, disminuyendo los niveles basales de NO, y permitiendo la translocación al núcleo de NF-κB y su unión a ADN. Como consecuencia, se incrementaría la expresión de la iNOS, tanto a nivel de mARN como de proteína, y produciendo las grandes cantidades de NO implicadas en la apoptosis.

7. El glutamato activa la nNOS, produciendo altas concentraciones de NO que estimulan la apoptosis e inhiben la activación de NF-kB.

8. Diferentes proteína quinasas, especialmente la PKA, PKC y ERKs, parecen estar implicadas en la apoptosis inducida por glutamato.

1. Modelo de mecanismos mitocondriales de apoptosis inducida por donadores de NO en células cromafines bovinas

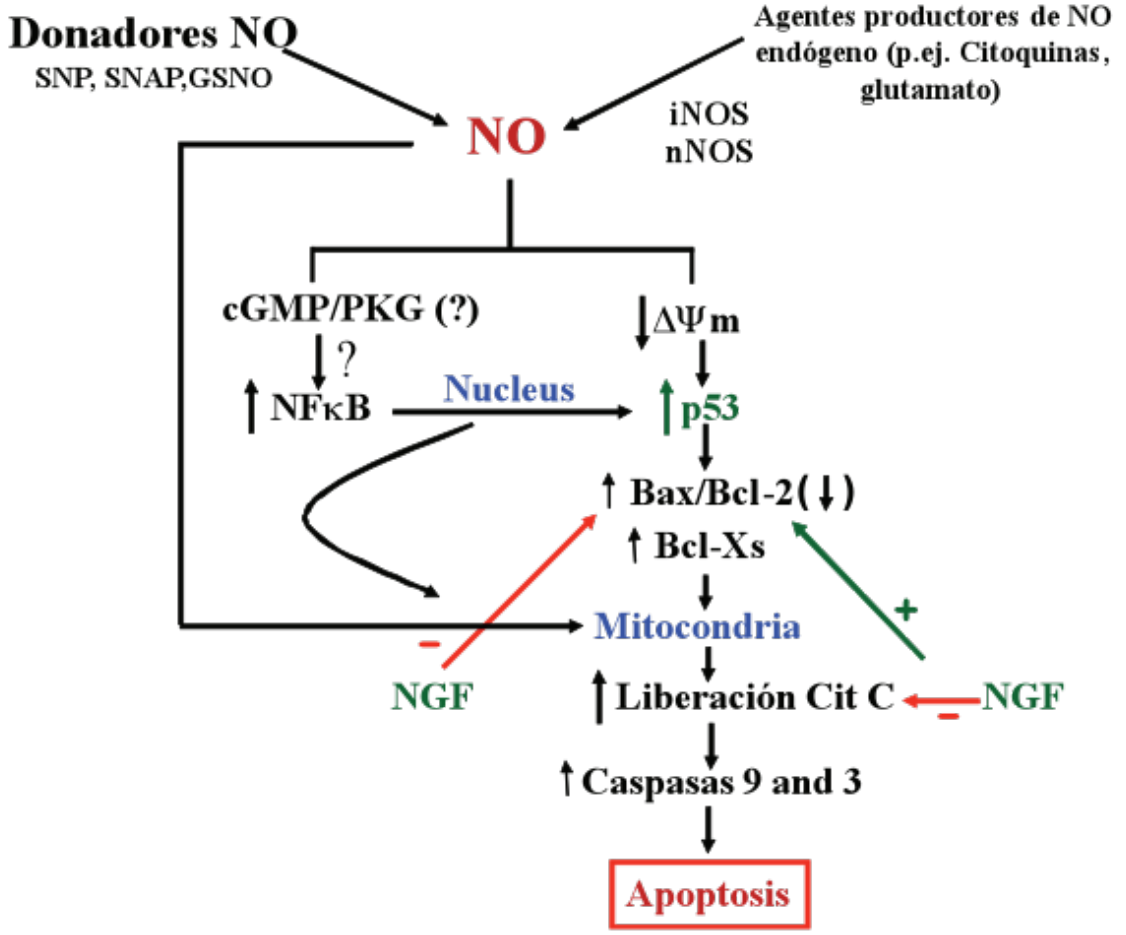

Figura 1. Modelo de mecanismos mitocondriales de apoptosis inducida por donadores de NO en células cromafines bovinas.
En la figura se resumen los mecanismos moleculares de los mediadores mitocondriales en la apoptosis inducida por donadores de NO en las células cromafines bovinas, descritos en este trabajo. Los donadores de NO median una disminución en el potencial de la membrana mitocondrial (Ψm), seguido de una disminución en la expresión de Bcl-2 (tal vez debida a la fosforilación de Bad, lo que permitiría su asociación con miembros apoptóticos de esta familia (Bax, BclXs)), un aumento en la liberación de citocromo c al citosol, la activación de las caspasas 3 y 9, y la muerte celular. Probablemente la inducción de la expresión de p53 sea uno de los primeros eventos en producirse tras el daño inicial, y podría ser el responsable de la regulación de las proteínas de la familia Bcl-2. A su vez, p53 podría regularse por el factor nuclear NF-κB. Estos procesos son bloqueados por NGF (Vicente et al. 2006). La generación de NO podría producirse de manera endógena por las isoformas de la NOS: nNOS o iNOS, tras un daño inducido por citoquinas o glutamato.

Conclusión final y modelos propuestos:
Como conclusión final, a la vista de nuestros resultados y a la luz de las evidencias existentes en la literatura con respecto a las acciones del NO exógeno sobre la vía mitocondrial apoptótica, y con respecto a las acciones del NO endógeno, basal o estimulado por citoquinas, glutamato y agonistas glutamatérgicos, sobre la apoptosis celular, proponemos los siguientes modelos para explicar sus efectos en las células cromafines:

2. Modelo propuesto para explicar el efecto del NO endógeno sobre la apoptosis de las células cromafines basal, mediada por citoquinas y glutamato.

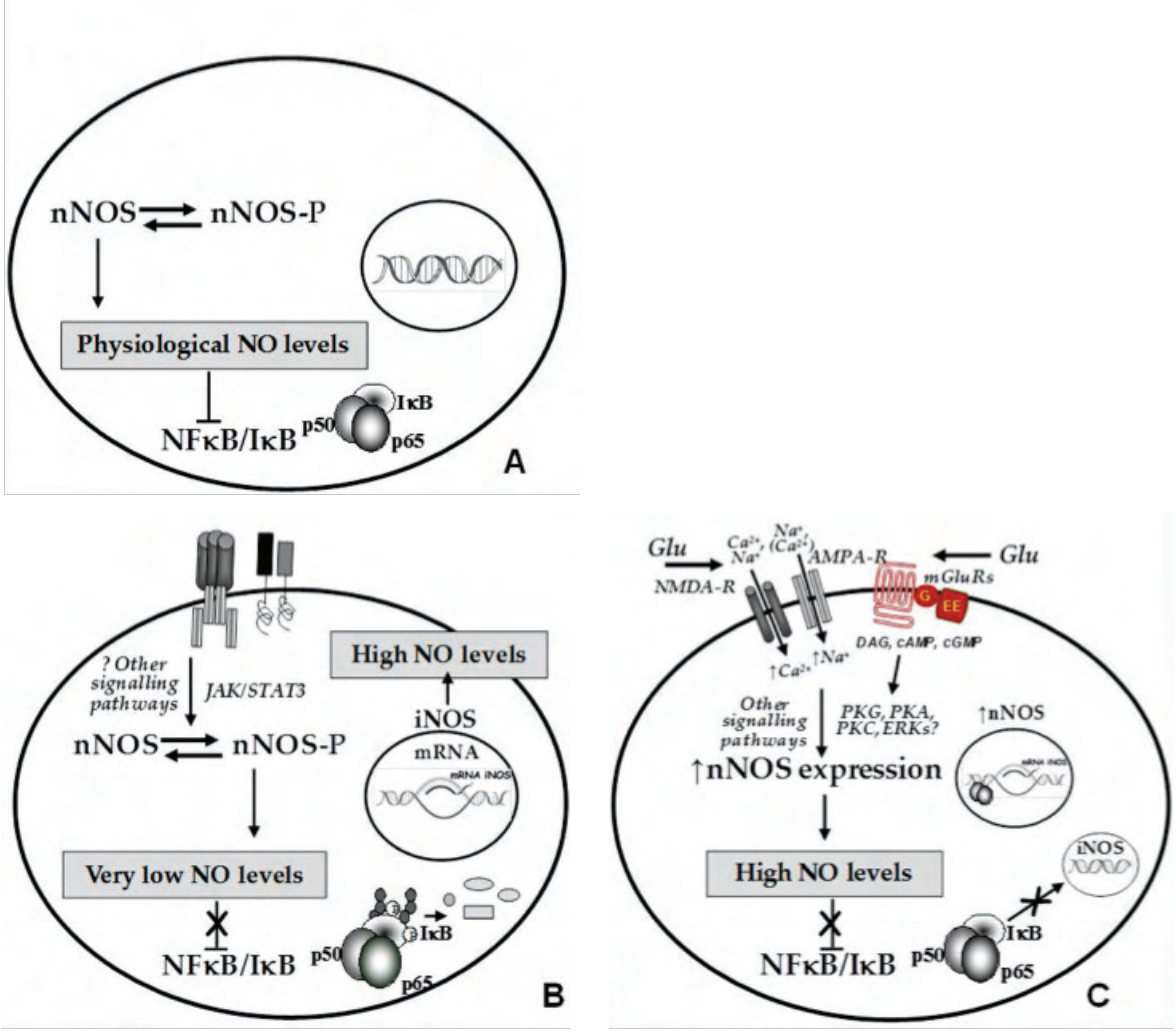

Figura 2: Modelo propuesto para explicar el efecto del NO endógeno sobre la apoptosis de las células cromafines basal (a) y mediada por citoquinas (b) y glutamato (c).
Este modelo implica que en condiciones basales, los niveles fisiológicos de NO generados por la nNOS bloquean la activación de NF-κB, impidiendo la apoptosis (a). Las citoquinas fosforilarían la nNOS, disminuyendo los niveles basales de NO y desbloqueando la activación de NF-κB, lo que conllevaría un incremento en la expresión de la iNOS y la generación de grandes cantidades de NO inductoras de apoptosis (b). El glutamato activaría la nNOS produciendo niveles de NO superiores a los basales que mantendrían bloqueado NF-κB evitando la activación de la iNOS y la producción de enormes cantidades de NO. El incremento en los niveles de NO y bloqueo de NF-κB contribuiría a su efecto apoptótico (c).

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X. Anexos

Se adjuntan a continuación otros artículos en los que ha participado la doctoranda y que se comentan en la discusión:

Vicente, S., Figueroa, S., Pérez-Rodríguez, R., González, M. P., & Oset-Gasque, M. J. (2005). Nitric oxide donors induce calcium-mobilisation from internal stores but do not stimulate catecholamine secretion by bovine chromaffin cells in resting conditions. *Cell calcium*, 37(2), 163–72. doi:10.1016/j.ceca.2004.09.002

Vicente, S., Pérez-Rodríguez, R., Oliván, A. M., Martínez Palacián, A., González, M. P., & Oset-Gasque, M. J. (2006). Nitric oxide and peroxynitrite induce cellular death in bovine chromaffin cells: evidence for a mixed necrotic and apoptotic mechanism with caspases activation. *Journal of neuroscience research*, *84*(1), 78–96. doi:10.1002/jnr.20853

Oliván, A M., Pérez-Rodríguez, R., Roncero, C., Arce, C., González, M. P., & Oset-Gasque, M. J. (2011). Plasma membrane and vesicular glutamate transporter expression in chromaffin cells of bovine adrenal medulla. *Journal of neuroscience research*, *89*(1), 44–57. doi:10.1002/jnr.22529

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Nitric oxide donors induce calcium-mobilisation from internal stores but do not stimulate catecholamine secretion by bovine chromaffin cells in resting conditions

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Abstract

The potential role of nitric oxide (NO) donors and peroxynitrites on both basal catecholamine (CA) secretion and modulation of calcium levels has been investigated in primary cultures of bovine chromaffin cells. NO donors did not modulate catecholamine secretion, while peroxynitrites induced a time dose-dependent increase in basal CA secretion. Two facts may explain the lack of these compounds on basal CA secretion. NO donors induce, on the one hand, an increase in intracellular calcium levels by depletion of internal IP_3-stores from endoplasmic reticulum. On the other hand, a small calcium influx through N-type voltage-dependent calcium channels (VDCC), which seem not to be coupled to exocytosis of adrenaline and noradrenaline in chromaffin cells. Both effects, calcium-mobilisation from internal stores and calcium entry through N-type VDCC are mediated by cGMP synthesis. In contrast, peroxynitrites induce an increase in basal CA secretion by both a decrease of intracellular catecholamine content and a toxic effect on cellular membrane. All these results, taken together, could explain contradictory results in the literature on the role of NO on basal catecholamine secretion and on modulation of intracellular calcium in chromaffin cells.
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Keywords: Nitric oxide; Chromaffin cells; Intracellular calcium-mobilisation; Peroxynitrite; Catecholamine secretion

1. Introduction

Nitric oxide (NO) is a highly reactive radical that plays an important role in the regulation of vascular tone, neuronal transmission and modulation of immunological and inflammatory reactions [1–3]. For the last 10 years, it has also been demonstrated that NO is involved in crucial physiological events such as the regulation of neurotransmitter release, and in pathological events underlying neurotoxicity and neurodegeneration [4–6].

NO is synthesised from L-arginine by nNOS in central and peripheral neural tissues. In adrenal chromaffin cells, Moro et al. [7] and Oset-Gasque et al. [8] demonstrated, by hystochemical and biochemical techniques, the presence of a constitutive NOS isoform, which has been identified as nNOS [9,10]. Moreover, Afework et al. [11] and Heym et al. [12] demonstrated the presence of NOS-containing fibres, closely associated with chromaffin cells.

NO has a well-established functional inhibitory role on CA secretion evoked by nicotine in chromaffin cells [8,13]. However, the nature of its functional role in the regulation of basal CA secretion is very controversial. Our previous studies showed that NO gas has an activatory effect on basal CA secretion in chromaffin cells [8], results which seem to be

Abbreviations: cADPR, cyclic adenosine diphosphate-ribose; ω-Aga, ω-agatoxin; BSA, bovine serum albumine; Caf, caffeine; $[Ca^{2+}]_i$, intracellular free calcium concentration; Ca^{2+}, calcium; CA, catecholamine; ω-Ctx, ω-conotoxine GVIA; $dONOO^-$, deactivated peroxynitrite; DMEM, Dulbecco's modified Eagle medium; Fura-2/AM, acetoxymethyl ester of fura-2; cGMP, 3′,5′-cyclic guanosine monophosphate; GSNO, *S*-nitrosoglutathione; Nife, nifedipine; NO, nitric oxide; $ONOO^-$, peroxynitrite; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; SNP, sodium nitroprusside; thaps, thapsigargin; VDCC, voltage-dependent Ca^{2+} channels

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confirmed by the fact that NO donors mediate an increase in intracellular calcium levels [9], thus implying the possibility of an activatory effect on CA secretion. On the contrary, studies from Machado et al. [14] indicating that NO donors decrease basal CA secretion in single chromaffin cells and recent results from our group showing that L-arginine inhibits basal CA secretion while NOS inhibitors enhance it, seem to demonstrate that NO, via cGMP formation, is involved in the maintenance of low basal CA secretion at resting level [10].

The discrepancies above make us think of a different mechanism for NO donors and NO gas action on basal CA secretion. NO donor's mechanism is possibly mediated by cGMP and might be possibly carried out by a Ca^{2+} mobilization from internal stores. NO gas mechanism may involve the participation of oxidative species of NO, like peroxynitrites.

In order to test these hypothesis and clarify the above indicated dissenting results, we measured the effect of NO donors and peroxynitrites on basal CA secretion and studied the mechanisms by which NO donors produce an increase in $[Ca^{2+}]_i$ in chromaffin cell without modifying CA secretion.

2. Experimental procedures

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were from GIBCO (BRL, UK), collagenase from *Clostridium histolyticum* (EC 3.4.4.19) was from Boehringer Mannheim S.A. (Barcelona, Spain). The NO donors sodium nitroprusside (SNP), *S*-nitroso-*N*-acetyl-penicillamine (SNAP), *S*-nitroso-gluthatione (GSNO), antibiotics, Fura-2/AM, cytosine arabinoside, (+)5-fluorodeoxyuridine (FDU), nifedipine and ω-CTX GVIA were from SIGMA Chemical (Madrid, Spain) and ω-Aga IVA. ODQ was obtained from Tocris Cookson (Bristol, UK). Amphotericin B was from ICN Ibérica S.A. (Barcelona, Spain). Cyclic GMP kit was from Amersham International (Buckinghamshire, UK). All other chemicals were reactive grade products from Merck (Darmstadt, Germany).

2.2. Cell isolation and culture

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Bader et al. [15] with minor modifications. Briefly, glands supplied by a local slaughterhouse were trimmed off fat, cannulated through the adrenal vein and washed with a free-calcium Krebs-HEPES solution (Locke medium) containing (mM): NaCl 154, KCl 5.6, glucose 5.6 and HEPES 5.0, pH 7.5, at 37 °C. Digestion was performed with a 0.2% collagenase plus 0.5% bovine serum albumin solution in the above medium. After digestion, chromaffin cells were removed, dispersed and purified through a Percoll gradient. Cell viability was checked by trypan blue exclusion, and chromaffin cell purity was assessed by the specific incorporation of neutral red to the cells. Both parameters were routinely higher than 90%.

Cells were suspended in DMEM containing 10% FCS, antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin, and 40 mg/ml gentamicin), and cytostatics (10 mmol/l FDU and 10 mmol/l cytosine arabinoside), and plated in 24 Costar cluster dishes at a density of 0.5×10^6 cells/well and used 3–5 days after plating.

2.3. Measurement of CA secretion

Cells were washed twice, at 10-min intervals, with 1 ml of a Krebs-HEPES solution (Locke medium) containing (mM): NaCl 140, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 11, ascorbic acid 0.5 and HEPES 15, pH 7.5, at 37 °C. This medium was removed from the wells and the cells were stimulated for 10 min at 37 °C with 0.5 ml of fresh Locke's medium (control cells) and Locke's medium containing different secretagogues, as indicated in each experiment. At the end of the test incubation period, the medium was taken and cells were lysed with 0.4 mol/l perchloric acid and scraped off the plates.

The incubation medium and the cell lysates were used for the determination of CA secretion and total CA content, respectively. Determination of CA secretion in both sample types, previously diluted and neutralised, was performed with an electrochemical detector (Metrohm 641 VA detector) adjusted to +580 mV and recorded (LKB recorder model 2210). A standard norepinephrine curve was used for calibration. Results were expressed as the percentages of CA release in the incubation medium over the total CA content (incubation medium plus pellet).

2.4. Measurement of nitrite production

Nitrites were determined as described Misko et al. [16], with minor modifications. This method is based on the measurement of the fluorescent product 1-(*H*)-naphtotriazole formed by the reaction of nitrites with 2,3-diaminonaphtalene (DAN) in acidic conditions.

Cells (0.5×10^6/condition) were stimulated for 10 min with 400 μl of the different NO donors mentioned above. After incubation, the medium was aspirated and 100 μl of the different samples were mixed with 50 μl of freshly prepared DAN (0.025 mg/ml in HCl 0.62 M) for 10 min in darkness. The reaction was stopped with 50 μl of NaOH 2.8N and the fluorescence was enhanced by incubating this solution in darkness for 5 min. The volume was completed to 1.5 ml with double-deionised water and the formation of 1-(*H*)-napthtotriazole was measured using a Perkin-Elmer LS50 fluorimeter with excitation and emission wavelengths set to 375 and 415 nm, respectively. The samples were calibrated with a standard curve of freshly prepared nitrites.

2.5. Measurement of intracellular levels of cGMP

Cells were stimulated for 10 min with the different NOS inhibitors as indicated in each experiment. Incubations were performed in the presence of 0.5 mmol/l IBMX in order to prevent cGMP degradation. The medium was removed and cells were lysed with 200 μl of 0.4 mol/l $HClO_4$. These cell lysates were neutralised with 1 mol/l KOH, centrifuged in an Eppendorf centrifuge and the supernatants used for the assay of cGMP levels. Intracellular cGMP measurement was performed by using the specific radioimmunoassay kits from Amersham, following manufacturer's instructions.

2.6. Measurement of $[Ca^{2+}]_i$

Cytosolic calcium was determined with the fluorescent indicator Fura-2/AM, as previously described [17]. The cells cultured on glass-cover slips at a density of 1.5×10^6 cells/coverslip, were washed twice with 1 ml of Locke's medium and loaded with 5 μM Fura-2/AM for 45 min at 37 °C. Excessive dye was removed by washing the cells twice with fresh Locke. Cells from coverslips were placed in a thermostatically controlled and well-stirred cuvette containing 1.5 ml of Locke. Fluorescence (excitation wavelengths = 340/380 nm and emission wavelength = 510 nm) was monitored in a Perkin-Elmer LS50 fluorimeter (slits 5 nm exc, 10 nm em). Some experiments were performed in a Locke's medium containing 200 nM $[Ca^{2+}]_o$, which was considered as a Locke's medium with low calcium. At the end of each experiment, 1% Triton X-100 was added to permeabilize the cells, allowing the dye to gain access to the extracellular Ca^{2+} (2.5 mM). This Ca^{2+} concentration saturated the dye and provided (F_{max}). To determine the minimum fluorescence signal (F_{min}), 20 mM of Tris base was added to raise the pH above 8.2, followed by 5 mM of EGTA, which reduced Ca^{2+} to less than 1 nM. The cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ was automatically calculated from fluorescence traces by using the Grynkiewicz et al. [18] equation:

$$[Ca^{2+}]_i = K_d \left(\frac{F - F_{min}}{F_{max} - F} \right) \left(\frac{SF_2}{SB_2} \right)$$

where F is the ratio between fluorescence values at 340 and 380 nm, SF_2 is the maximum fluorescence at 380 nm and SB_2 is the minimum fluorescence at 380 nm. The equilibrium dissociation constant (K_d) for the complex $[Ca^{2+}]$-Fura was 224 nM.

2.7. Measurement of LDH release

For these studies, bovine chromaffin cells grown in 24-well culture dishes (3–7 days) were stimulated with NO donors, peroxynitrite or NO gas for 10 min. Then, medium was taken and cells were lysed by scrapping them in 250 μl of a buffer containing 0.5% (v/v) Triton X-100 in a 0.1 M potassium phosphate buffer, pH 7.4. The buffer was then removed after centrifugation at 10,000 rpm for 5 min and LDH activity was determined in both the medium and lysis buffer in the presence of 0.5 mM pyruvate and 0.15 mM NADH by the spectrophotometric method of Vassault [19]. % of LDH release = (LDH in medium)/(LDH in medium + LDH in lysis buffer).

2.8. Statistics

Data shown are means ± S.E.M. of at least three experiments each one performed in duplicate. Statistical significance was estimated with Student's t test for unpaired observations. A P value of <0.05 was considered significant. Fitting of concentration–response curves for estimation of EC_{50} and IC_{50} values were made by weighted non-linear regression of minimum squares, using logistic curves.

3. Results

3.1. NO donors do not modulate basal catecholamine secretion

We assessed the effects of the NO donors SNAP, SNP and GSNO (1–100 μM) on both basal CA secretion and NO production. All the NO donors assayed induced a dose-dependent release of NO, measured by its ability to produce nitrites (Fig. 1). The most potent effect was obtained with SNAP (Fig. 1B): concentrations of 100 μM increased approximately 10-fold the basal levels, followed by GSNO (Fig. 1C) and SNP (Fig. 1A), which increased NO formation in about 4- and 2-fold the basal level, respectively.

When we assessed the effects on CA secretion, at the same doses than in the study of NO production, we were unable to find any statistically significant change on basal CA secretion (Fig. 1). These data indicated a lack of correlation between CA secretion and nitrite production by NO donors.

3.2. Peroxynitrites induce an increase in basal CA secretion

Chromaffin cells were treated with 100 μM of both active peroxynitrites and peroxynitrites deactivated by keeping them at 37 °C for 10 min in Locke's medium, so that the solution contained the degradation products of peroxynitrites (nitrites, nitrates and H_2O_2) and the vehicle (NaOH). Incubations were performed for different periods, as shown in Fig. 2. At 10 min and doses of 100 μM only the active peroxynitrites induced an increase in CA secretion which was about five times higher than the basal levels. The fact that deactivated peroxynitrites did not significantly increase the basal CA secretion demonstrated that the effect of peroxynitrites was specific and not due to the decomposition products or the vehicle.

After studying the time-dependent effect of peroxynitrites, we analysed their effects on CA secretion at doses from 1 μM

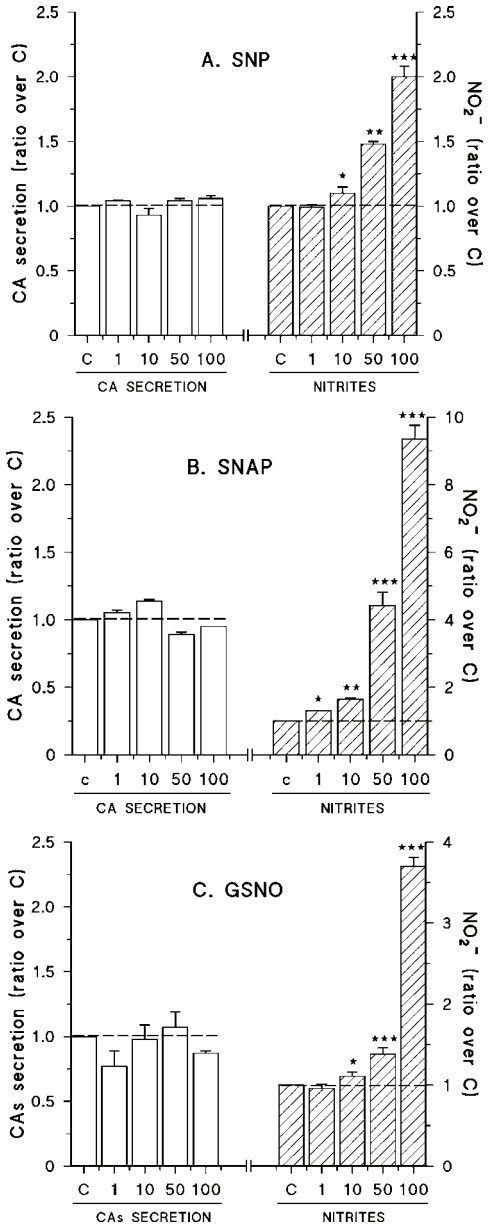

Fig. 1. Effect of (A) SNP, (B) SNAP, (C) GSNO on catecholamine secretion and NO production. Cells were incubated for 10 min with the indicated concentrations of NO donors and both the CA secretion and NO production were measured as described in Section 2. The results were expressed as a ratio over their own control values ($C_{\text{basal CA secretion}} = 4.37 \pm 0.40\%$ over total cellular content (t.c.c.); $C_{\text{basal NO production}} = 0.27 \pm 0.09$ nmol/mill cells). Data are mean ± S.E.M. of three determinations performed in duplicated ($n = 6$). The statistical significances compare the effect of NO donors over their own basal controls ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's t test).

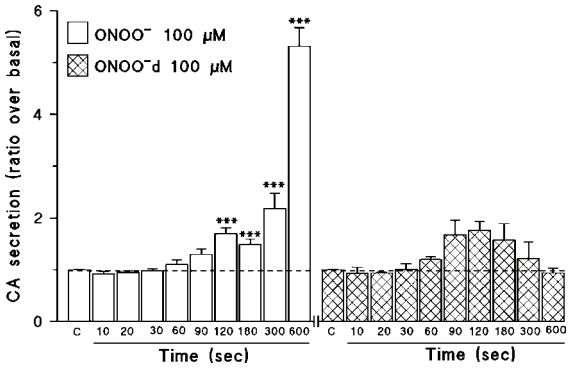

Fig. 2. Time course of peroxynitrites effect on basal CA secretion. Cells were treated for 10 s to 10 min with 100 µM peroxynitrites or deactivated peroxynitrites, and the CA secretion was measured as described in Section 2. The results were expressed as the ratio over their own control values ($C_{\text{basal CA secretion}} = 4.03 \pm 0.2\%$ over t.c.c.). Data are the mean ± S.E.M. of three determinations performed in duplicate ($n = 6$). The statistical significances compare the effect of peroxynitrites and deactivated peroxynitrites over their own basal controls ($^{***}P < 0.001$; Student's t test).

to 5 mM for 10 min, time at which CA secretion was maximal. Peroxynitrites induced a dose-dependent increase in CA secretion that was maximal at the dose of 1 mM and represented about 12 ± 3.2 times the basal levels (Fig. 3A), while deactivated peroxynitrites did not induced CA secretion, at any used dose. The EC_{50} for peroxynitrites on CA secretion was 510 ± 98 µM. On the other hand, peroxynitrites diminished the CA content in chromaffin cells. This inhibition showed a IC_{50} of 398 ± 70 µM (Fig. 3B). The increase in basal CA secretion induced by peroxynitrites correlates significantly with a decrease in intracellular CA content (Fig. 3B, inset).

The fact that NO donors produce no changes in CA secretion and that active peroxynitrites induce both an increase in CA release and a fall in the total intracellular content of CAs could be explained by a combination of damage in the cell membrane and inhibition of CA synthesis. In order to check the first possibility, we measured the effect of peroxynitrites on LDH release, as a parameter of membrane integrity, and compared this effect with the one obtained in the case of NO donors and NO gas. Peroxynitrites 1 mM produced a significant increase in LDH release at 10 min, while the NO donors, at the same dose, had no effect (Table 1). However, NO gas at high doses also produces a small increase in LDH release. In addition of that, total CA content (sum of release + cell content) is significantly reduced in the presence of $ONOO^-$ (Fig. 3, inset) and not in the presence of $_{d}ONOO^-$ or NO donors (data not shown). These results indicate that the differences in CA secretion observed for peroxynitrites and NO donors could be due to both a toxic effect of peroxynitrites upon the cell membrane integrity and an inhibition in CA biosynthesis, facts that also occur when cells are challenged with high doses of NO gas.

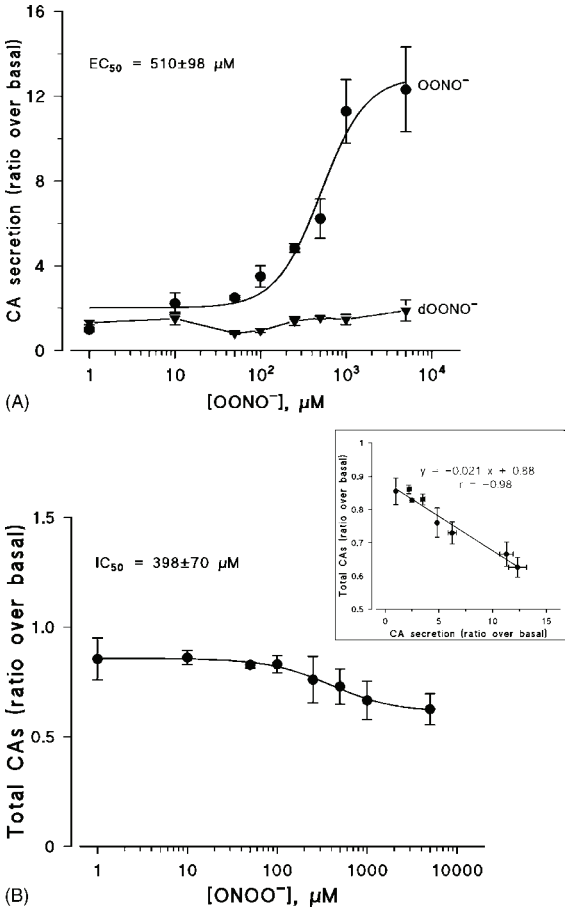

Fig. 3. Effect of peroxynitrites and deactivated peroxynitrites on basal CA secretion. (A) Dose–response curves and (B) decrease in intracellular CA content. Cells were stimulated for 10 min with the indicated concentrations of peroxynitrites and deactivated peroxynitrites, and the CA secretion and content were measured as indicated in Section 2. The results were expressed as a ratio over their own control values ($C_{\text{basal CA secretion}} = 4.08 \pm 0.3\%$ over total catecholamine content). The data are the mean ± S.E.M. of three determinations performed in duplicate ($n = 6$). Statistical analysis of correlation was performed by linear regression by minimum squares method ($P < 0.001$; Student's *t*-test).

3.3. NO donors induce a calcium-mobilisation from IP_3-dependent internal stores mediated by cGMP

As indicated from previous results from our group, NO donors increase basal $[Ca^{2+}]_i$ in resting conditions [9]. As this work indicates that these compounds do not induce any increase in basal CA secretion, we tried to clarify this discrepancy by studying the effect of NO donors on $[Ca^{2+}]_i$. The stimulation of chromaffin cells with different doses of SNP and SNAP (1–500 µM) produced a dose-dependent increase in cytosolic $[Ca^{2+}]$ in both with and without calcium medium which oscillates between 150 and 320 nM. Maximal effects for SNAP and SNP were obtained at a dose of 100 µM and they were of a similar magnitude in both conditions (300 ± 45 nM and 275 ± 52 nM in the presence or the absence of extracellular calcium, respectively for SNAP and 220 ± 31 nM and 190 ± 49 nM for SNP (Fig. 4)). These results suggest that NO donors induce an increase in $[Ca^{2+}]_i$ mainly by a calcium-mobilisation from internal stores.

Table 1
Effect of peroxynitrites and NO donors on LDH liberation

Conditions	% LDH (ratio over basal)
Control	1 ± 0.08
SNP 1 mM	1.05 ± 0.22^{ns}
SNAP 1 mM	1.10 ± 0.30^{ns}
P 1 mM	10.43 ± 1.2***
Pd 1 mM	3.3 ± 0.8***
NO gas 1 mM	2.25 ± 0.7***

Cells were treated for 10 min with 1 mM SNP, SNAP, pure (P) or deactivated peroxynitrites (Pd) and NO gas. The measurement of LDH was performed in the supernatants and the pellets as indicated in Section 2. $C_{\text{basal release of LDH}} = 1.23 \pm 0.5\%$. ns: non-significant.
*** $P < 0.001$; Student's *t* test.

To analyse which intracellular compartment could participate in the release of calcium evoked by NO donors, the

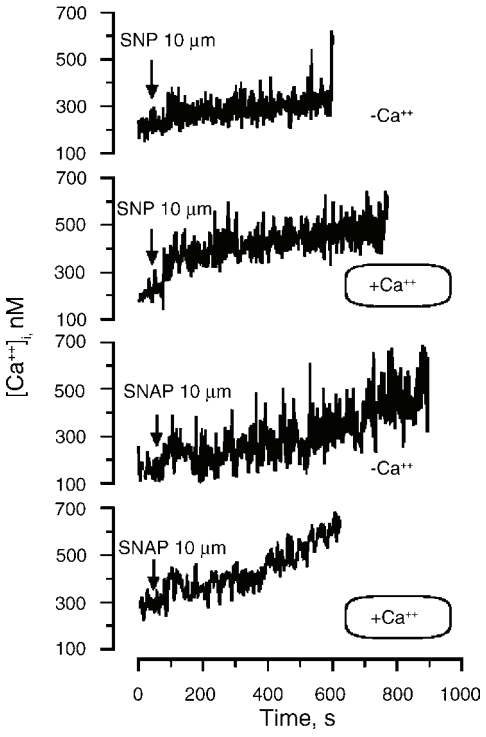

Fig. 4. Effect of NO donors on $[Ca^{2+}]_i$. Cells were treated for 10 min with SNP and SNAP in a medium with ($+Ca^{2+}$) and without ($-Ca^{2+}$) extracellular Ca^{2+} at the indicated doses. The measurement of the variations of the $[Ca^{2+}]_i$ was performed as indicated in Section 2 and results were expressed as nM. Figures are representative records from at least three different experiments of four to five different cell batches.

Table 2
Effect of some compounds acting on Ca^{2+} release from internal stores on basal $[Ca^{2+}]_i$ (A) and effect of NO donors on increases in $[Ca^{2+}]_i$ evoked by the above compounds (B)

Conditions	$[Ca^{2+}]_i$ (nM)		
	(A) Basal effect	(B) 100 μM SNAP	100 μM SNP
Control (−Ca^{2+})	–	280 ± 34	155 ± 20
100 μM angiotensin II	350 ± 50	112 ± 21***	64 ± 12***
1 μM thapsigargin	180 ± 30	20 ± 6***	66 ± 15***
10 μg/ml oligomycin	99 ± 6	19 ± 4***	33 ± 8***
4 mM caffeine	250 ± 38	230 ± 29	154 ± 24

Cells were incubated for 5–10 min in the absence of extracellular calcium with the indicated compounds (A), then stimulated with 100 μM SNAP or SNP (B) and calcium was measured as indicated in Section 2. The data, expressed in nM, are the mean ± S.E.M. of four determinations performed in duplicate. The statistical significances compare the effect of NO donors on Ca^{2+} levels in the absence of the Ca^{2+}-mobilising compounds (control) with those obtained in the presence of these compounds.
*** $P < 0.001$; Student's *t* test.

effects of various compounds depleting different pools of Ca^{2+} internal stores were studied. These agents were angiotensin II, an agent that induces the formation of IP_3; thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase; oligomycin, an inhibitor of the F_oF_1ATP synthase which produces an intracellular ATP depletion, and caffeine, a compound that interferes with the uptake and storage of Ca^{2+} by sarcoplasmic reticulum. All these compounds induced an increase in calcium levels (Table 2A). Pre-treatment of chromaffin cells with these agents for 5 min, followed by a 10-min treatment with SNP or SNAP diminished the increase in $[Ca^{2+}]_i$-evoked by NO donors. This inhibitory effect was very high in the case of angiotensin II, thapsigargin (60–90%) and oligomicyn (80–90%) (Table 2B and Fig. 5), and smaller for caffeine (about 5–15%) (Table 2B and Fig. 5). All these results seem to demonstrate that NO donors induce Ca^{2+} mobilisation from internal stores mainly from endoplasmic reticulum sensitive to IP_3.

The possible implication of Ca^{2+} in the effect of peroxynitrite-induced increases in CA secretion was also tested. Peroxynitrites produced a large linear increase in $[Ca^{2+}]_i$ at low doses (50–100 μM) (data not shown) at which they do not significantly increase CA secretion. However, at higher doses, peroxynitrites don't modify $[Ca^{2+}]_i$, that indicating chromaffin cell death. Thus, the possible involvement of $[Ca^{2+}]_i$ in peroxynitrite-induced cell damage was discarded.

NO donors induce an increase in intracellular cGMP levels between 10- and 20-fold (75–150 pmol/10^6 cells) basal levels at a dose of 100 μM. In order to know whether their effects on $[Ca^{2+}]_i$ are mediated by the activation of guanylate cyclase, we assayed the effect on $[Ca^{2+}]_i$ of the permeable analogue of cGMP, 8-Br-GMPc. This compound induced a dose-dependent increase in intracellular calcium levels, maximal at a dose of 10 μM, of about 2.5-fold the basal levels (275 ± 23 nM). This calcium increase was not abolished in an external calcium-free medium (Fig. 6A) but was

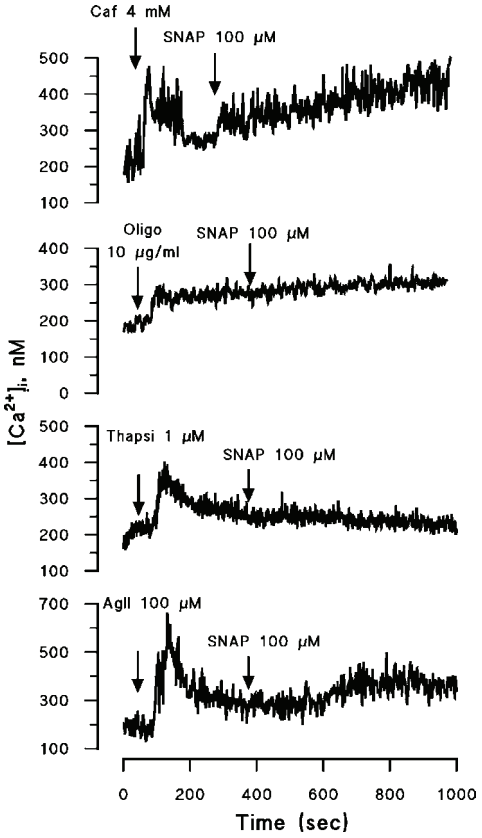

Fig. 5. Effect of NO donors on mobilisation of Ca^{2+} from internal stores. Cells were treated for 10 min with 100 μM SNAP in a medium without extracellular Ca^{2+}, in the presence of angiotensin II (Ang II), thapsigargin (Thapsi), oligomycin (Oligo) and caffeine (Caf) at the indicated doses. The measurement of the variations of the $[Ca^{2+}]_i$ was performed as indicated in Section 2. The results were expressed as nM. Figures are representative records of at least three different experiments performed in different cell batches.

completely blocked in the presence of thapsigargin and angiotensin II while caffeine only blocked about a 40% of this effect (Fig. 6B). In order to confirm whether the effect of NO-donors on intracellular Ca^{2+} mobilisation was mediated by cGMP, we tested the ability of ODQ, a specific inhibitor of soluble a guanylate cyclase, on these SNP-mediated effects. Results in Table 3 indicate that this compound was able to significantly inhibit the release of Ca^{2+} from both, not depleted and previously depleted by thapsigargin or angiotensin II, IP_3-sensitive stores. Taken together, these results indicate that the calcium depleting effect of NO donors on IP_3-sensitive stores is mediated by activation of soluble guanylate cyclase.

3.4. NO donors also induce a small calcium entry through N-type calcium channels mediated by cGMP

Although $[Ca^{2+}]_i$ increases induced by NO donors in both the presence and absence of extracellular Ca^{2+} were very

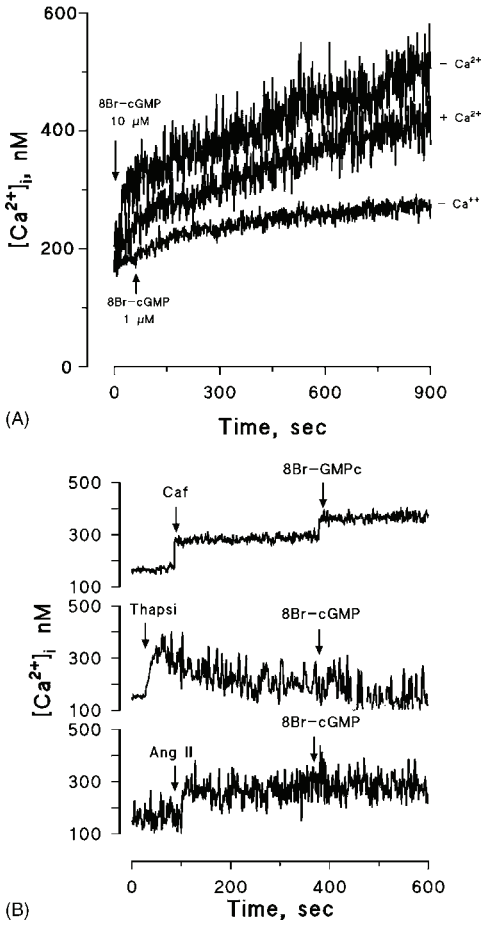

Fig. 6. Effect of cGMP on (A) basal $[Ca^{2+}]_i$ and (B) Ca^{2+} mobilization from internal stores. Cells were treated for 10 min with the indicated doses of 8-Br-cGMP in a medium with ($+Ca^{2+}$) and without extracellular Ca^{2+} ($-Ca^{2+}$), alone (A) or in the presence of 4 mM caffeine (Caf), 100 μM angiotensin II (Ang II) or 1 μM thapsigargin (Thapsi) (B). The measurement $[Ca^{2+}]_i$ was performed as indicated in Section 2. The results were expressed as nM. Figures are representative records of at least three different experiments performed in different cell batches.

similar, we observed that these responses were sometimes a little higher in the presence of extracellular Ca^{2+}. Thus, in order to discard or consider the possibility of a Ca^{2+} entry through voltage-dependent calcium channels (VDCC) we studied the effect of different calcium channel blockers on the Ca^{2+} entry mediated by NO donors. The pre-incubation of chromaffin cells with either 10 μM verapamil or 1 μM nifedipine (selective blockers of L-type VDCC) or with 300 nM ω-agatoxin IVA (a selective blocker of P/Q VDCC) for 5 min, prior to the incubation with 100 μM SNP or SNAP, did not inhibit the $[Ca^{2+}]_i$ increase induced by these NO donors (Table 4). However, the pre-incubation with 1 μM ω-conotoxin GVIA (a selective blocker of N-type VDCC) inhibited around 30–35% the response of NO donors (Table 3). An

Table 3
Effect of ODQ on SNP-induced Ca^{2+} mobilization in bovine chromaffin cells

Conditions	$[Ca^{2+}]_i$ (nM)	
	−10 μM ODQ	+10 μM ODQ
Control ($-Ca^{2+}$)/SNP	156 ± 22	57 ± 18***
+100 μM Ang II/SNP	67 ± 20	23 ± 8***
+1 μM thapsi/SNP	68 ± 18	17 ± 5***

Cells were incubated for 5 min without ODQ or with 10 μM ODQ in the absence of extracellular calcium, then with Locke (control) or angiotensin or thapsigargin for 5 min and finally with 100 μM SNP. Cytosolic calcium was measured as indicated in Section 2. The basal effect of Ang II and Thapsi on $[Ca^{2+}]_i$ was not affected by ODQ pre-treatment (data not shown). The data, expressed in nM, are the mean ± S.E.M. of three determinations performed in duplicate. The statistical significances compare the effect of SNP after pre-treatment with Ang II or Thapsy in the absence or presence of ODQ.
*** $P < 0.001$; Student's t test.

Table 4
Effect of VDCC blockers on increases in $[Ca^{2+}]_i$ induced by NO donors

Conditions	$[Ca^{2+}]_i$ (nM)	
	100 μM SNP	100 μM SNAP
Control ($+Ca^{2+}$)	217 ± 41	320 ± 44
10 μM verapamil	180 ± 44	289 ± 38
1 μM nifedipine	170 ± 46	263 ± 33
1 μM ω-conotoxin GVIA	130 ± 26***	211 ± 29***
300 nM ω-agatoxin IVA	250 ± 38	325 ± 25

Cells were incubated for 5 min in the presence of extracellular calcium with the indicated compounds, then stimulated with 100 μM SNAP or SNP and calcium was measured as indicated in Section 2. The data, expressed in nM, are the mean ± S.E.M. of four determinations performed in duplicate. The statistical significances compare the effect of NO donors on Ca^{2+} levels in the absence of the VDCC blockers (Control) with those obtained in the presence of these compounds.
*** $P < 0.001$; Student's t test.

inhibition of about 45 ± 10% of $[Ca^{2+}]_i$ by 1 μM ω-conotoxin GVIA was also shown in the case of cell stimulation with 8Br-cGMP, this effect being almost fully reversed (80 ± 10%) by 10 μM ODQ (data not shown). Taken together, these results indicate that NO donors are able to induce a small Ca^{2+} entry through N-type VDCC mediated by the activation of soluble guanylate cyclase.

4. Discussion

Nitric oxide is an important intracellular messenger involved in the regulation of neurosecretion in chromaffin cells. Previous work from our group demonstrated that NO modulates CA secretion evoked by nicotine in a dose-dependent manner: high doses of nicotine inhibit CA secretion in a dose-dependent manner through a mechanism involving cGMP [8].

However, while there is a general agreement with the fact that NO decreases CA secretion evoked by high depolarising stimuli like ACh, nicotine and high KCl, results are contradictory on the effect of NO on basal CA secretion and $[Ca^{2+}]_i$.

Thus, our previous results show that NO gas induces a large stimulation of basal CA secretion [8] and that NO donors evoke $[Ca^{2+}]_i$ increases in a high percentage ($62 \pm 4\%$) of whole population of single chromaffin cells, being the response to NO donors between 30 and 50% of that of 20 μM nicotine [9]. Since about 70% of adrenergic cells responded to NO donors and only 45% of noradrenergic cells induced $[Ca^{2+}]_i$ increases, we stated that the main NO cellular target are adrenergic cells [9]. Nevertheless, our last work showing that nNOS inhibitors induce a dose-dependent increase in basal CA secretion blocked by L-arginine, [10], supports an inhibitory effect of NO in CA secretion.

In order to clarify these apparently contradictory results, in this paper we focused on the effect of NO donors and peroxynitrites on basal CA secretion and basal $[Ca^{2+}]_i$ levels.

Our results demonstrate that NO donors and peroxynitrites have different effects on basal CA secretion, while NO donors do not modify basal CA secretion, peroxynitrites evoke CA secretion in a dose-dependent manner. This difference could be due to a toxic effect of peroxynitrites, since these compounds produce a large release of LDH and NO donors do not. The fact that NO donors and peroxynitrites have different effects on CA secretion seems very interesting in order to explain the wide range of effects that have been ascribed to NO in bovine chromaffin cells, including our previous data [8] in which we postulated the increase of CA secretion evoked by NO gas. Thus, the activatory effect of NO gas on basal CA secretion could be mediated, at least in part, by the formation of peroxynitrites in chromaffin cells. This could be supported by the following observations: (1) NO gas-induced increases of basal CA secretion are partially due to a decrease in the CA content accompanied by small increases in LDH release. (2) Peroxynitrites decrease both TH activity and expression (data not shown), thus reducing CA content. So, a partially toxic effect could explain the increase in basal CA secretion produced by NO gas. Therefore, our results with peroxynitrites seem to explain, at least in part, the possible discrepancy between the effect of NO gas and the NO donors on basal CA secretion.

The lack of response on basal CA secretion of NO donors has not only been shown in chromaffin cells, but also in PC12 cells and other species by other authors. Ward et al. [20] described that SNP was unable to induce CA secretion in dog chromaffin cells, and Naganuma et al. [21] showed that SNP and SNAP had no effect on CA secretion in PC12 cells in spite of they induced an increase in both cGMP and intracellular calcium levels [22,23]. This result is similar to ours as NO donors induced increases in basal Ca^{2+} levels without affecting the basal CA secretion ([9]; present work).

What is most important in this work is the mechanism through which these NO donors do not modulate the basal CA secretion. We demonstrate that the increase in calcium levels, evoked by NO donors, may be mainly mediated by the release of calcium from internal stores, this effect being not enough to induce the CA secretion. Naganuma et al. [22] and Clementi et al. [23] described that the store implicated in calcium-mobilisation in PC12 cells is the one sensitive to ryanodine and caffeine. However, in our case, the store mainly implicated in this mobilisation seems to be the IP_3-sensitive from endoplasmatic reticulum. Depletion of calcium from internal IP_3-sensitive stores with NO has been already proved by us in an indirect way. Thus, we had shown that NO gas induced an increase in IP_3 formation [8] which may explain the subsequent increase in intracellular calcium levels evoked by NO gas and NO donors. In this paper, we have demonstrated that the increase in Ca^{2+} mobilisation evoked by NO donors is inhibited if the cells are previously treated with angiotensin II (a Ca^{2+}-mobilisator compound through an IP_3-sensitive mechanism) or with thapsigargin (an inhibitor of the Ca^{2+}-ATPase of the endoplasmic reticulum). However, Ca^{2+} mobilisation from caffeine sensitive-stores seems not to be involved since the effect of caffeine on Ca^{2+} mobilisation induced by NO donors was very small compared with that obtained with the first agents. From data obtained with oligomycin, mobilisation of calcium by NO might also be coupled to some mitochondrial mechanisms, since the pretreatment of cells with oligomycin also abolished the increase in calcium-mobilisation produced by NO donors. However, as oligomycin is a blocker of the F_oF_1-ATPase (ATP synthase), and no activity of this compound on Ca^{2+} uniporter nor Ca^{2+}/Na^+ exchanger has been demonstrated, the increase in $[Ca^{2+}]_i$-evoked by oligomycin, and, as a consequence, its inhibitory effect of NO donors-evoked calcium-mobilisation, must be a consequence of an inhibition of Ca^{2+}-ATPase from endoplasmic reticulum due to the ATP depletion rather than to any mitochondrial-specific mechanism.

NO donors-induced calcium-mobilisation from IP_3-dependent internal stores is mediated, at least in part, by the activation of soluble guanylate cyclase (sGC), since analogous of cGMP mimicked the effect of NO and sGC inhibitors blocked specifically this effect. So, we could conclude that, as already described, NO mediates its physiological functions through the formation of cGMP.

We observed that NOS inhibitors induce CA secretion [10], whereas NO donors do not inhibit it. How could we explain that? What is the real mechanism acting in there? It is likely that the technique for measuring total CA secretion is not sensitive enough to detect small decreases on CA secretion induced by NO donors. It is also probable that NO induces opposite effects on the different CAs (NA, A and DA) release; therefore the activated release of one of the CA could mask an inhibitory effect upon the others. Indeed, recent data not yet published of our group measuring the release of different CAs by HPLC techniques indicate that NO donors induce inhibition on adrenaline and noradrenaline and activation on dopamine (DA) release, which could finally mask the former inhibitory effect. Ca^{2+} mobilisation from IP_3-dependent internal stores could be responsible of store-operated Ca^{2+} channels (SOCCs) or N-type VDCC opening and thus Ca^{2+} entry through these channels could be mediating the DA release. This mechanism could not be able to induce neither A nor NA release, probably because NO has an inhibitory

effect on Ca^{2+} entry through L-type VDCC [24] and the main VOCC involved in the control of CA secretion in chromaffin cells are L- and Q-type Ca^{2+} channels due to their closer localisation to the secretory sites [25], and their higher proportion in bovine chromaffin cells (P/Q (50%) > L (30%) > N (20%)) [26]. The role of NO/cGMP pathway in the induction of DA secretion has been recently demonstrated in PC12 cells by microdialysis techniques, and both in PC12 and chromaffin cells, is mediated by Ca^{2+} entry through nifedipine-insensitive channels [27].

The exact mechanism through which NO inhibits basal CA secretion is not completely understood, although on the basis of literature some mechanisms would be proposed such as: (1) a slow-down in the exocytotic machinery measured by amperometric detection of exocytosis of CAs in single cells [14]; (2) a depression of the single Ca_V l L-channels measured by patch-clamp techniques [24]; and (3) an activation of Ca^{2+}-activated K^+ channels [28]. In addition, recent results from our group show that this inhibitory effect could be related to the increases on the ATP release probably from compartments distinct from chromaffin granules (Oset-Gasque et al., data not published). ATP released could inhibit the activation of calcium currents, thus controlling the release of CAs. This inhibitory mechanism has been described in chromaffin cells by Gandía et al. [29], and it implies a regulatory mechanism through which the CA secretion is controlled to low levels.

In conclusion, results from present paper indicate that contradictory effects of NO on CA secretion and on $[Ca^{2+}]_i$ described in the literature may be explained by different evidences: (1) peroxynitrites are involved in some effects of NO donors, (2) the mechanism involved in NO donors-$[Ca^{2+}]_i$ increase is a mobilisation of calcium internal stores or a Ca^{2+} entry through N-VDCC, and (3) the different effects of NO on different catecholamine release and the insensibility of some techniques to detect these inhibitory effects. These results also reinforce the idea that the use of physiological substrate, arginine, or NOS inhibitors [10] could be a better experimental approach than NO donors when studying the role of the L-arginine/NO/cGMP pathway on neurotransmitter and hormone secretion.

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Nitric Oxide and Peroxynitrite Induce Cellular Death in Bovine Chromaffin Cells: Evidence for a Mixed Necrotic and Apoptotic Mechanism With Caspases Activation

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Treatment of chromaffin cells with nitric oxide (NO) donors (SNP and SNAP) and peroxynitrite produces a time- and dose-dependent necrotic and apoptotic cell death. Necrotic cell death was characterized by both an increase in lactate dehydrogenase and ATP release and changes in nuclei and cell morphology (as seen with fluorescence microscopy analysis with propidium iodide and Hoechst 33342). Apoptotic cell death was characterized by nuclear fragmentation and presence of apoptotic cell bodies, by a decrease in DNA content, and by an increase in DNA fragmentation. Treatment of chromaffin cells with lipopolysaccharide (LPS) or cytokines (interferon-γ, tumor necrosis factor-α) resulted only in apoptotic cell death. Apoptotic effects of NO-inducing compounds were specifically reversed, depending on the stimuli, by the NO scavenger carboxy-PTIO (CPTio) or by the NOS inhibitors L-NMA and thiocitrulline. NO-induced apoptotic death in chromaffin cells was concomitant to a cell cycle arrest in G_0G_1 phase and a decrease in the number of chromaffin cells in the G_2M and S phases of cell cycle. All NO-producing compounds were able to induce activation of caspase 3 and cytochrome c release, and specific inhibitors of caspase 3 and 9, such as Ac-DEVD-CHO (CPP32) and Ac-Z-LEHD-FMK, respectively, prevented NO-induced apoptosis in chromaffin cells. These results suggest that chromaffin cells could be good models for investigating the molecular basis of degeneration in diseases showing death of catecholaminergic neurons, phenomenon in which NO plays an important role. © 2006 Wiley-Liss, Inc.

Key words: nitric oxide; nitric oxide synthase; cellular death; chromaffin cells; glutamate; peroxynitrite; apoptosis; necrosis; adrenal medulla

Nitric oxide (NO) is a messenger molecule involved in several processes, including smooth muscle relaxation, neurotransmission, tumor cells, and bacterial killing (Moncada et al., 1991; Nathan, 1992; Garthwaite and Boulton, 1995). NO exerts its physiological effects by regulating the guanylate cyclase activity and possibly by mild, partially reversible covalent protein modifications, such as S-nitrosylation (Stamler, 1994; Martínez-Ruiz and Lamas, 2004). However, induction of a high NO-output system in response to cytokines or a massive production of NO following accumulation of the excitatory neurotransmitter glutamate (Dawson et al., 1991; Lipton et al., 1993) can result in cell killing. Neurons (Stamler et al., 1992), pancreatic β-cells (Bergman et al., 1992), or macrophages (Albina et al., 1993) seem to be particularly sensitive to NO toxicity.

Necrosis and apoptosis are distinct mechanisms of cell death, with very different characteristics. Necrosis is caused by toxic or traumatic events, with passive cell swelling, injury to cytoplasmic organelles including mitochondria, and quick collapse of internal homeostasis. Necrosis leads to membrane lysis, release of cellular contents, and resulting inflammation (Kerr and Harmon, 1991; Schwartz et al., 1993). In contrast, apoptosis is an active process of neuronal cell destruction with specific defining morphologic and molecular features, such as membrane blebbing, chromatin condensation, and DNA fragmentation (Kerr et al., 1972; Bursch et al., 1992). Although in some systems NO reacts with other radicals and causes necrotic cell death, in others progressive

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intra- or extracellular generation of NO may cause apoptosis (Albina et al., 1993; Messmer et al., 1995; Ankarcrona et al., 1994).

Enzymatically produced NO can undergo a large variety of nonenzymatic reactions, e.g., with cellular thiols, metals, or superoxide (O_2^-). Products arising from these secondary reactions, particularly peroxynitrite ($ONOO^-$), formed from NO· and O_2^-, have been implicated in cortical neuronal and PC12 cell apoptosis (Bonfoco et al., 1995; Estévez et al., 1995).

Oxidative stress that results in generation of free radicals has been implicated in a final common pathway for neurotoxicity in a wide variety of acute and chronic neurological diseases (for review see Murphy, 1999). In these disorders, excessive stimulation of excitatory amino acid receptors may trigger the production of free radicals. Particularly, neurotoxicity associated with overstimulation of N-methyl-D-aspartate (NMDA) receptors is thought to be mediated by excessive Ca^{2+} influx, resulting in a series of neurotoxic events (Lipton and Rosemberg, 1994). One of these events is the activation of nitric oxide synthase (NOS) and the resulting production of NO (Dawson et al., 1991). Another neurotoxic event is the stimulation of phospholipase A_2 or mitochondrial Ca^{2+} overloading, leading to generation of superoxide anion (O_2^-). NO· can react with O_2^- to form peroxynitrite ($ONOO^-$), resulting in a dose-dependent neuronal damage (Lipton et al., 1993).

Adrenal chromaffin cells are neurosecretory cells derived from the neural crest. In rats, these cells proliferate extensively before birth and, in a milder way, also throughout life (Malvaldi et al., 1968; Tischler et al., 1989). Adult rat chromaffin cells proliferation is regulated, in vivo, by neurally derived signals and, in vitro, by stimulation with peptidic growth factors or by activation of adenylate cyclase or protein kinase C that mimics the effects of neurotransmitters in adrenal medullary nerve endings (Tischler et al., 1994). Therefore, these cells could be used as a model for development of the nervous system.

In bovine chromaffin cells, the presence of a constitutively expressed neuronal NOS (nNOS) has been demonstrated by both biochemical and immunocytochemical methods (Oset-Gasque et al., 1994, 1998; Schwarz et al., 1998; Vicente et al., 2002). In addition, the presence of NOS fibers closely associated with those choline acetyltransferase (ChAT)-positive fibers innervating rat chromaffin cells has also been shown (Holgert et al., 1995; Tanaka and Chiba, 1996). In these cells, the L-arginine/NO/cGMP pathway has an important inhibitory role in both basal and acetylcholine (ACh)-stimulated catecholamine (CA) secretion (Schwarz et al., 1998; Vicente et al., 2002).

Tumoral chromaffin PC12 cell lines have been widely studied as models of necrotic and apoptotic cell death induced by different stimuli (such as serum and trophic factors deprivation or NO challenge; Chung et al., 1999; Bal-Price and Brown, 2000). However, there is little evidence in the literature supporting the possibility that chromaffin cells could undergo physiological apoptotic death (Jordan et al., 2000, 2002), and it is not known whether NO, apart from its biological actions, could have a neurotoxic effect in these cells. Therefore, we studied the effects of NO on necrotic and apoptotic cell death in chromaffin cells. This study was extended to $ONOO^-$, to determine the possible participation of this toxic anion on NO-induced cell death.

Our results demonstrate that treatment of chromaffin cells with NO donors and/or cytokines, the last inducing NO formation by NOS activation, produced mainly an apoptotic cell death, whereas treatment with $ONOO^-$ mediated both necrosis and apoptosis, depending on the dose and time of stimulation. Therefore, we assessed that the intensity of original insult could be related to the chromaffin cell death pathway. These results seem to demonstrate that chromaffin cells could constitute a good model for studying the molecular mechanisms underlying catecholaminergic neuron degeneration.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium, fetal calf serum, HEPES, and RNAse A were purchased from Gibco BRL (Poole, United Kingdom); collagenase from *Clostridium histolyticum* (EC 3.4.4.19) was supplied by Boehringer Mannheim S.A. (Barcelona, Spain). The NOS inhibitor S-methyl-L-thiocitruline hydrochloride (thiocitrulline) was obtained from Tocris Cookson (Bristol, United Kingdom). Antibiotics, cytosine arabinoside, 8-fluoro-desoxiuridine (FDU), lipopolysaccharide (LPS), interferon (IFN)-γ, superoxide dismutase (SOD), Hoesch 33342, neutral red, propidium iodide, olygomicin, rotenone, and nerve growth factor were from Sigma (Madrid, Spain). Amphotericin B was from ICN Ibérica S.A. (Barcelona, Spain). The luciferin-luciferase (L-L) reagent was from BioOrbit (1243-118 ATP Biomass Kit) and the test Kinesis 50 (Kinesis 50, 470-0023) from Bio-Rad Laboratories (Richmond, CA); SITO-13 and hydroethidine were from Molecular Probes (Eugene, OR). Anticytochrome c monoclonal antibody and the caspase 3 fluorogenic substrate peptide Ac-DEVD-amc were supplied by BD PharMingen International (Becton Dickinson Co., Mountain View, CA). Peroxynitrite and carboxy-PTIO were purchased from Alexis Biochemicals (Lausanne, Switzerland), and 2,3-diaminonaphtalene (DAN), CPP32, and Z-LEHD-FMK were from Calbiochem-Novabiochem Co (La Jolla, CA). All other chemicals were reactive-grade products from Merck (Darmstadt, Germany).

Chromaffin Cell Culture and Drug Treatments

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Vicente et al. (2005). Cell viability was checked by trypan blue exclusion, and chromaffin cell purity was assessed by specific incorporation of neutral red to these cells. Both parameters were routinely higher than 90%.

Cells were suspended at a 0.5×10^6/ml density in Dulbecco's modified Eagle's medium (DMEM) containing 10%

fetal calf serum, antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, and 40 mg/ml gentamicin), and cytostatics (10 mmol/liter fluorodeoxyuridine and 10 mmol/liter cytosine arabinoside), plated in 24 Costar cluster dishes, and used 3–7 days after plating. Three to seven days after plating, cells were washed three times with DMEM and exposed to NO donors or cytokines, by adding the different compounds to culture medium in concentrated form and mixing very gently. The cultures were then incubated as indicated for the figures. In the case of peroxynitrite treatment, to minimize reactions of this compound with bicarbonate and other components in culture medium, cells were incubated in 1 ml DMEM for 1–2 min before addition of a single bolus of this compound. Peroxynitrite was quickly added on one edge of the culture dish, and buffer was quickly swirled for 5 sec to distribute the peroxynitrite within the dish. Five minutes after peroxynitrite exposure, buffer was removed and replaced by 10% fetal calf serum (FCS) supplemented culture medium. What we used as a control treatment was decomposed peroxynitrite (Pd), obtained by adding peroxynitrite to the buffer before addition to the cells.

Quantitation of Cytotoxicity

Cytotoxicity was estimated by quantization of lactate dehydrogenase (LDH) and ATP content and efflux from cells into incubation medium after different times of treatment.

Measurement of LDH Activity. For these measurements, 3–7 days aged bovine chromaffin cells grown in 24-well culture dishes were stimulated with NO donors, peroxynitrite, or cytokines in DMEM medium at short (15 min) or long (24 hr) intervals. Then, medium was collected and cells were lysed by scraping them in 250 μl of a buffer containing 0.5% (v/v) Triton X-100 in a 0.1 M potassium phosphate buffer, pH 7.4. The buffer was then removed after centrifugation at 10,000 rpm for 5 min, and LDH activity was determined in both the medium and the lysis buffer in the presence of 0.5 mM pyruvate and 0.15 mM NADH by the spectrophotometric method of Vassault (1983). Antagonists or blockers were added 5 min before agonists. [Percentage of LDH release = LDH in medium (LDH in medium + LDH in lysis buffer)].

Determination of ATP Release and Content. ATP release in response to different NO-generating compounds was determined by using the luciferin-luciferase (L-L) reagent (BioOrbit, 1243-118 ATP Biomass Kit) according to the manufacturer's instructions. Briefly, after stimulation of cells (0.5×10^6) with different NO-generating compounds or vehicle in 250–500 μl of a Krebs-HEPES solution (Locke medium) containing (mM) NaCl 140, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 5.5, ascorbic acid 0.5, and HEPES 10, pH 7.5, for variable times, supernatants were collected and cells frozen in 200 μl Locke's medium at –80°C. After freezing, 25 μl of supernatants or 25 μl of cell pellets diluted 1/10 in the Locke's medium were added to 25 μl of ATP Releasing Reagent and 50 μl of 0.1 mM Tris-acetic buffer, pH 7.75, and incubated for 5 min at 37°C in a BioOrbit 1251 Luminometer with continuous stirring. Then, 25 μl of ATP monitoring reagent (luciferin-luciferase) was added and the ATP content determined by the increase in luminescence. For calibrating the light signals, 10 pmol of ATP dissolved in Tris-acetic buffer was injected into each sample, and luminescence signal was determined. ATP cellular content was expressed as pmol ATP/10^6 cells and ATP release as percentage of ATP released over total ATP cellular content.

Measurement of Superoxide Production. Superoxide production was monitored with 5 μM dihydroethidine (HEt; Molecular Probes) as previously described (Jordan et al., 2000). Background was subtracted, and fluorescence was recorded at different times (4 hr and 24 hr) by using an excitation filter of 535 nm and an emission filter of 635 nm in a spectrofluorimeter (Bio-Tek FL 600). Fluorescence was recorded every 20 sec during a 7-min period. Linear regression of fluorescence data was calculated for each condition, and the slope (a) of the best fitting line ($y = ax$) was considered as an index of O_2^- production.

NO Measurements

For measuring amounts of NO released by different NO-generating compounds, nitrites (the final stable products of NO) were determined by using the spectrofluorimetric method of Misko et al. (1993). This method is based on the reaction of nitrite with 2,3-diaminonaphtalene (DAN) under acidic conditions to form 1-(H)-naphthotriazole, a fluorescent derivative product. After cell (0.5×10^6) stimulation with tested compounds dissolved in Locke's medium during different times, 100 μl of supernatants were mixed with 10 μl of freshly prepared DAN (0.05 mg/ml in 0.62 M HCl). After a 10-min incubation period, in the dark at 37°C, the reaction was stopped with 5 ml of 2.8 N NaOH. Formation of the fluorescent 2,3-diaminonaphthotriazole was measured with a Perkin Elmer LS-50 fluorimeter. Excitation and emission wavelengths were set to 365 and 450 nm, respectively. The DAN reagent was protected from light. Nitrite standards (more than 98% purity; purchased from Sigma) were routinely freshly made, dissolved in double-deionized water, and kept on ice prior to use.

Analysis of DNA Fragmentation

Internucleosomal DNA fragmentation was assessed by agarose gel electrophoresis analysis of fragmented DNA by a modified version of the method of Lyons et al. (1992). The dishes ($1–2 \times 10^6$ cells) were washed twice with ice-cold PBS, and lysis was accomplished with 1 ml of 20 mM EDTA, 0.5% Triton X-100, 5 mM Tris-HCl, pH 8.0. Dishes were gently shaken for 15 min at 4°C. After that, intact nuclei were pelleted and eliminated by centrifugation at 500*g* for 10 min, and the supernatant was centrifuged at 25,000*g* at 4°C for 30 min. DNA from the supernatant was precipitated with ethanol 2.5 V at –80°C, pelleted by microcentrifugation at 4°C for 15 min, dried, and resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer). Then, it was incubated with 0.1 mg/ml RNAse A during 30 min at 37°C and with 0.25 mg/ml proteinase K for 2–3 hr at 37°C to eliminate RNA and proteins. DNA was purified by phenol-chloroform extraction, precipitated with ethanol at –80°C, and resus-

pended in TE buffer. Then, DNA, stained with 0.5 μg/ml of ethidium bromide, was electrophoresed in a 1.5% agarose gel.

Confocal Microscopy Analysis

Morphology of chromatin from apoptotic and intact nuclei was detected by confocal microscopy analysis of chromaffin cells attached to plates, incubated for 15 min with SYTO 13 (3 μM) and neutral red (NR; 1%) in PBS. SYTO 13 is a permeable dye that selectively stains nucleic acids of all cells, emitting green fluorescence, and NR was used to stain selectively amide phenolic compounds (catecholamines), emitting red fluorescence. The use of both colorants together allowed us to determine whether there were necrotic or apoptotic processes taking place in the chromaffin cell population. Apoptotic chromaffin cells were distinguished for having green SYTO 13-stained nuclei inside red-stained cytoplasm. Thus, by using this method, we can distinguish between intact nuclei and necrotic/apoptotic nuclei. MRC 1000 confocal microscopy (Bio-Rad, Hempstead, United Kingdom) was used, and digital images were printed by using laser graphics.

Fluorescence Microscopy Analysis

Morphology of nuclei and cells was detected by fluorescence microscopy analysis of chromaffin cells attached to plates and incubated for 15 min with 0.005% propidium iodide (PI) and 1 μM Hoechst 33342 in PBS. Only necrotic cells and late apoptotic cells can be labelled by PI, because PI only enters cells that have lost their plasma membrane integrity. Hoechst 33342 specifically stains DNA, being able to enter all cells. Viable cells were identified by blue fluorescence resulting from Hoechst staining excited by UV light. Injured (necrotic and apoptotic) cells were identified by the penetration and intercalation of PI in DNA. Cells were visualized in a Nikon Diaphot (Japan) fluorescence microscope.

Flow Cytometric Analysis

Analysis of DNA content and cell cycle was performed in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). DNA was stained with PI by using the Bio-Rad reagent kit (Kinesis 50, 470-0023; Bio-Rad, Richmond, CA), following the manufacturer's protocol. Measurements were carried out using a Double Discriminator Module in order to discriminate doublets. Ten thousand cells were acquired per sample. After that, the percentage of cells with DNA content lower than 2C was calculated, as well as the percentage of cells in the G_0G_1, S, and G_2M phases of the cell cycle, by using Multicycle software (Phoenix Software, Mountain View, CA).

Fluorometric Analysis of Caspase 3 Activity

After 24 hr incubation with NO donors or cytokines, the culture medium was decanted, and the cells (0.5×10^6/well) were quickly washed twice with PBS wash buffer (140 mM NaCl, 2.7 mM KH_2PO_4, pH 7.5) and then resuspended in a lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaPPi, and 0.5% Triton X-100; 1 million/ml). Cell lysates were repeatedly passed through a syringe (25-gauge needle) until most of the cell membrane was disrupted and were centrifuged at 10,000*g* for 10 min at 4°C. Activity of caspase 3 was measured by using the fluorogenic substrate peptide DEVD-amc (66081; BD Biosciences PharMingen). Cytosolic protein (~50 μg) was incubated with 20 μM substrate peptide in a 150-μl final volume of incubation buffer [20 mM HEPES, 10% glycerol, 2 mM dithiothreitol (DTT), pH 7.5] at 37°C for 2 hr. The release of fluorogenic amc was measured by using a Perkin-Elmer fluorimeter (excitation at 380 nm, emission at 460 nm). A negative control (lysis buffer without cells) was used throughout.

Cytochrome c Determination

For analyzing cytochrome c release, chromaffin cells (5×10^6) were resuspended in 250 mM sucrose, 25 mM Tris/HCl, pH 6.8, 2.5 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, 50 μg/ml trypsin inhibitor, and 40 μg/ml aprotinin. Samples were centrifuged at 13,000*g* for 3 min at 4°C. Supernatants (containing the cytosolic fraction) and pellets (containing the mitochondrial fraction resuspended in 120 μl of a mitochondrial lysis buffer containing 20 mM HEPES, pH 7.6, 1 mM EDTA, 300 mM KCl, 5% glycerol, 0.2% Triton X-100, 5 mM DTT, 0.2 mM phenylmethylsulfonylfluoride, 40 μg/ml leupeptin, 50 μg/ml trypsin inhibitor, and 40 μg/ml aprotinin) were denatured, separated by SDS-12% polyacrylamide gel electrophoresis (50 μg protein/condition), and transferred to Immobilon-P membranes, as described above. In all cases, immunoblots were blocked in TTBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dried milk and were incubated overnight with the primary antibody (diluted 1:1,000 in TTBS + 0.5% nonfat dried milk). After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (1:5,000 in TTBS + 0.5% nonfat dried milk) for 2 hr, and the blot was developed with the ECL system (Amersham, Buckinghamshire, United Kingdom).

Statistical Analysis

Data were expressed as mean ± SEM values of three or four independent experiments with different cell batches, each performed in duplicate or triplicate. Statistical comparisons were assessed by using one-way analysis of variance (ANOVA; Scheffe's F-test), followed in some instances by a two-way ANOVA. Differences were accepted as significant at $P < 0.05$.

RESULTS

NO-Producing Agents and Peroxynitrite Induce a Decrease in Chromaffin Cell Viability

When chromaffin cell cultures were treated with SNP, an NO donor; LPS; or cytokines (IFNγ and TNFα), no significant cell damage was observed at 15 min (Table I). However, 24 hr treatment of chromaffin cells with SNP or cytokines, alone or in combinations, produced a cell deterioration detected by an increase in LDH release ranging from 1.5 to 2 times basal levels for cytokines and SNP, respectively. The cytokine-induced increases in LDH release were specifically reverted by the NOS inhibitors L-NMA and thiocitrulline at a

TABLE I. Effect of SNP and Cytokines on LDH Release at Short and Long Incubation Times[†]

Conditions	% LDH release (ratio over control) A) 15 Min	B) 24 Hr
Control	1 ± 0.08	1 ± 0.05
SNP 1 mM	1.05 ± 0.02 ns	2.53 ± 0.20^{***000}
LPS 10 ng/ml	1.13 ± 0.03 ns	1.48 ± 0.02^{***000}
IFNγ 50 Ui/ml	1.18 ± 0.04 ns	1.32 ± 0.03^{***00}
TNFα 10 nM	1.10 ± 0.02 ns	1.25 ± 0.01^{**00}
LPS + IFNγ	1.17 ± 0.05 ns	1.76 ± 0.06^{***000}
LPS + IFNγ + TNFα	1.20 ± 0.06 ns	1.62 ± 0.04^{***000}
P	6.53 ± 0.65***	3.42 ± 0.40^{***000}

[†]Chromaffin cells were stimulated for 15 min (A) or 24 hr (B) with SNP, P (peroxynitrite), or different cytokines alone or in combinations. Cells stimulated with P for 24 hr were washed after 15 min of incubation. Results, expressed as %LDH release ratio over control, are mean ± SEM of three experiments each performed in duplicate. (C 15 min = 2.71% LDH release over total cell content; C 24 hr = 5.60 ± 0.04%). Statistics compare the effect of different agents over control (ns, non significant; $^{**}P < 0.01$, $^{***}P < 0.001$) and the effect of time exposure for each condition ($^{00}P < 0.01$, $^{000}P < 0.001$; two-way ANOVA test).

1 mM concentration (Fig. 1A,B) and were directly correlated with NO amounts released by these compounds (Fig. 1A, inset). These results indicate that cytokine-induced cytotoxicity at 24 hr is due to NO formation. In the same way, dose-dependent increases in LDH release induced by SNP were specifically reversed by 1 mM CPTio (a NO scavenger) at all studied doses (Fig. 2A), suggesting that cellular death induced by this compound is due to NO. However, in the case of SNP, CPTio inhibition of increase in LDH release induced by SNP was dependent on SNP concentration. That is, whereas CPTio was able to block completely LDH release induced by low doses of SNP (1–10 μM), it was able to inhibit only about 70% of LDH release induced by high doses of SNP (0.5–1 mM). These results indicate that LDH release induced by high SNP doses could be due to peroxynitrite formation. This idea was confirmed by results obtained with SOD, a superoxide scavenger, which was able to reduce by 30–40% LDH release induced by high doses of SNP (Fig. 2A).

Peroxynitrite ($ONOO^-$) is generally considered the terminal neurotoxic species formed from NO· and O_2^-. Direct addition of 1 mM peroxynitrite, as a single bolus,

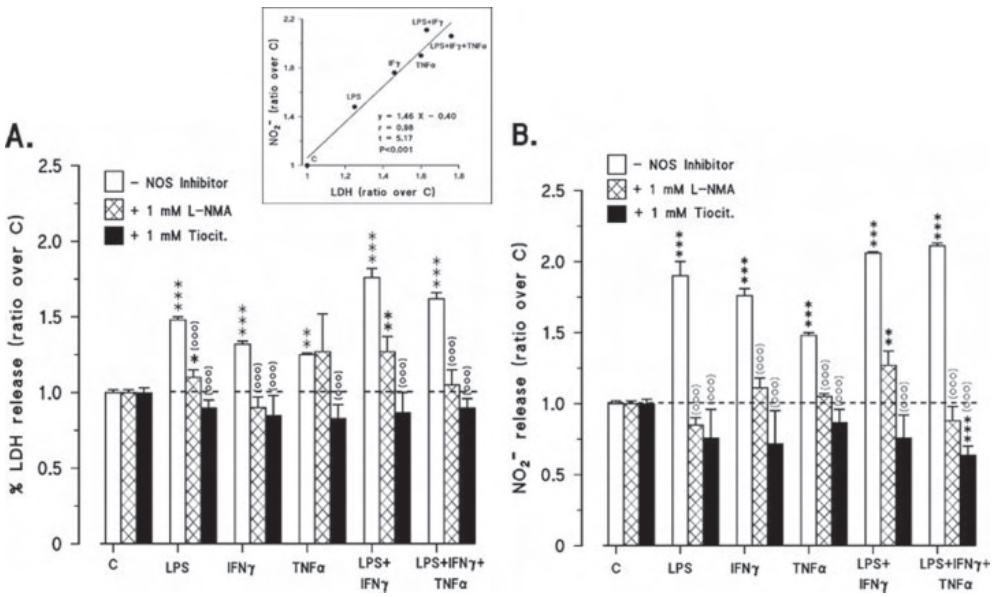

Fig. 1. Effect of LPS and cytokines on LDH release (**A**) and NO (nitrite) release (**B**) at long incubation times: reversion of effect by NOS inhibitors. Bovine chromaffin cells were treated during 24 hr with the indicated concentrations of cytokines, alone or in combination, and both LDH secretion and NO production were measured as described in Materials and Methods. Results were expressed in ratios of percentage LDH release or NO_2^- production over their control values. Data are mean ± SEM of three determinations performed in duplicated (n = 6). The statistical significances compare the effect of cytokines in the absence (open bars) and presence of 1 mM L-NMA (hatched bars) or 1 mM L-thiocitrulline (solid bars) over their own basal controls at $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ and the effect of NOS inhibitors on the increases in LDH release induced by each compound at $^{\circ\circ\circ}P < 0.001$ (two-way ANOVA). *Inset* shows linear correlation between LDH and NO (NO_2^-) induced by tested cytokines and their combinations. ($C_{basal\ LDH\ secretion}$ = 13.80% ± 0.50% over total cellular content; $C_{basal\ NO\ production\ (nitrite)}$ = 0.58 ± 0.04 nmol/million cells); LDH_{Tio} = 13.10% ± 0.40%; $NO_{2\ Tio}^-$ = 0.52 ± 0.02 nmol/million cells; $LDH_{L\text{-}NMA}$ = 13.52% ± 0.51%; $NO_{2\ L\text{-}NMA}^-$ = 0.48 ± 0.06 nmol/million cells.

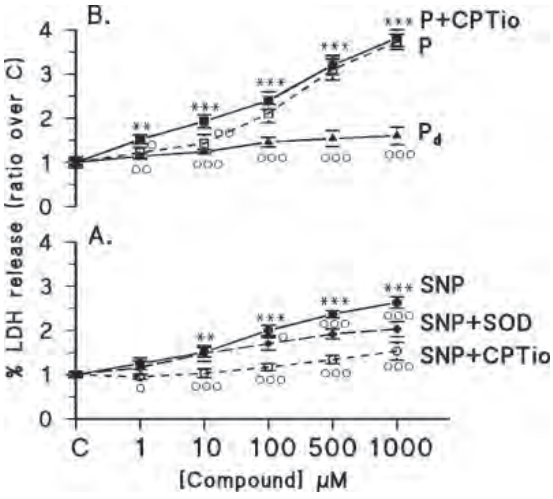

Fig. 2. Effect of SNP and peroxynitrite on LDH release at long incubation times: reversion by CPTio and SOD. Bovine chromaffin cells were treated for 24 hr with 1 mM SNP (**A**) or 1 mM P (peroxynitrite) or Pd (deactivated peroxynitrite; **B**) at the indicated concentrations, in the absence or presence of 100 µM CPTio or 50 UI/ml SOD, and LDH secretion was measured as described in Materials and Methods. Results, expressed in ratios of percentage LDH release over control values, are mean ± SEM of five determinations performed in duplicate (n = 6). Statistics compare the effect of SNP or P with the control value ($C_{\text{basal LDH secretion}}$ = 19.6% ± 0.04% over total cellular content; $^{**}P < 0.01$, $^{***}P < 0.001$) and the effect of CPTio and SOD on SNP and P-induced increase in LDH release ($^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$; two-way ANOVA).

to chromaffin cells produced a rapid time-dependent increase in LDH release of about six times the basal levels in 15 min (Table I). These results indicate the induction of an extensive necrotic cell damage characterized by acute cellular swelling and lysis, which could be observed rapidly after treatment. However, 5–10 min of incubation with P and subsequent washes, which eliminate detached cells, no longer showed that acute necrotic death. Thus, in these cells, after 24 hr of treatment with P, P-induced LDH release was lower than in cells incubated during acute, shorter times (these results being related to controls; Table I). P-induced LDH release at 24 hr was dose dependent and was greater than LDH release induced by SNP at the same doses. However, unlike SNP-induced LDH release, CPTio was able to reverse P-induced cellular death only at high doses of P (0.01–1 mM; Fig. 2B), indicating that this compound does not allow us to distinguish completely between the contributions of NO and P to NO donors-induced LDH release (at least at low levels of P formation), establishing the requirement of carrying out experiments with SOD (to trap O_2^- anions and therefore prevent P formation) when one want to study the contribution of NO and P to the NO donors effect. Nonetheless, P decomposition products (Pd) had no significant effect on cell viability at 24 hr (Fig. 2B), indicating the specificity of the P effect.

NO Induces an Increase in ATP Release but Not in ATP Depletion in Chromaffin Cells

ATP depletion has been associated with neurodegeneration. To determine whether NO-induced toxicity was parallel to a decrease in ATP levels, cultured cells were challenged to 1 mM SNP treatment in the presence or absence of 100 µM CPTio or 50 UI/ml SOD. SNP increased ATP release by about 10 times the control levels and diminished ATP cellular content by about 25% with respect to basal levels (Table II), these data suggesting the existence of necrotic cell death. However, insofar as total intracellular ATP content (obtained by adding intracellular ATP + released ATP) was not diminished with respect to control, the possibility of a concomitant apoptotic cell death was not excluded. To test whether the increase in ATP release by SNP could be due to NO release or to peroxynitrite formation, SNP-induced ATP release was measured in the presence of CPTio, an NO scavenger, or SOD, an enzyme that traps O_2^- anions, inhibiting P formation. These two compounds were able to reverse both SNP-induced effects, the increase in ATP release and the reduction in intracellular content (Table II), indicating that both NO and P species could be mediating the effects of SNP. To examine the possible NO donors' peroxynitrite formation, we monitored the O_2^- production induced by SNP and SNAP by a microfluorimetric assay as stated in Materials and Methods and compared these results with veratridine (and other products)-generated O_2^- production. Results in Table III show that chromaffin cells have a basal O_2^- production rate of 3.85 ± 0.21 a.f.u. min^{-1}, which does not vary significantly with time of incubation. By contrast, 50 µM veratridine (which prevents inactivation of voltage-dependent Na^+ channels), rotenone (which inhibits complex I in the mitochondrial respiratory chain), and oligomycin (an inhibitor to F_0F_1 ATP synthase) caused a time-dependent increase in the rate of O_2^- production, reaching values ranging from 2 to 4.5 times basal. O_2^- production by NO donors (SNP and SNAP) was also time-dependent, although smaller than with the above compounds, because the increase resulting from SNP and SNAP ranged between 1.2 and 1.9 times the basal values, indicating that, although NO· plus O_2^- formation of peroxynitrite participates in NO-donor-generated toxicity, this participation is small with respect to total cytotoxic effect of these compounds.

NO Donors, Cytokines, and Peroxynitrite Induce Morphological Chromaffin Cell Alterations Typical of Both Necrotic and Apoptotic Cellular Death

The microscopic observation of SNP- and cytokine-treated cells stained with Hoechst 33342 and PI indicates that a vast percentage of viable cells (round, blue nuclei in Fig. 3A′) displayed a homogeneous round

TABLE II. Effect of SNP on ATP Release and ATP Intracellular Content[†]

Conditions	ATP release (% over total cellular content)	Cell ATP (pmol/10^6cells)	Total ATP (pmol/10^6 cells)
Control	2.24 ± 0.12	605.41 ± 29.20	619.32 ± 30.14
SNP 1 mM	24.10 ± 0.29★★★	452.32 ± 18.42★★★	602.94 ± 10.45
CPTio 100 μM	2.34 ± 0.33	607.30 ± 18.64	621.92 ± 24.60
CPTio + SNP	11.65 ± 0.83★★★	557.73 ± 12.74★★	630.41 ± 33.12
SOD 50 Ul/ml	2.04 ± 0.28	601.34 ± 27.28	614.09 ± 28.23
SOD + SNP	10.63 ± 0.90★★★	523.17 ± 31.49★★	589.61 ± 35.94

[†]Chromaffin cells were treated for 24 hr with normal Locke (control), 100 μM CPTio, or 50 Ul/ml SOD in the absence and in the presence of 1 mM SNP. Then, medium was collected and cells lysed. After that, ATP release and content were measured as indicated in Materials and Methods. Results are mean ± SEM of three experiments each performed in duplicate. Statistical significances were calculated by one-way ANOVA test, tested conditions vs. the corresponding controls (★★$P < 0.01$, ★★★$P < 0.001$).

TABLE III. Effect of NO-Producing Compounds on Superoxide Anion Production at Short and Long Incubation Times[†]

	A) 4 Hr		B) 24 Hr	
Conditions	Slope (y = ax)	Ratio	Slope (y = ax)	Ratio
Control	3.85 ± 0.21	1.00 ± 0.05	3.80 ± 0.65	1.00 ± 0.17
Veratridine 50 μM	9.82 ± 1.91★★★	2.55 ± 0.50	16.38 ± 2.23★★★	4.31 ± 0.59^{000}
SNP 1 mM	4.84 ± 0.53★	1.26 ± 0.14	7.10 ± 0.60★★★	1.87 ± 0.16^{000}
SNAP 1 mM	4.64 ± 0.47★	1.21 ± 0.12	6.51 ± 0.95★★★	1.71 ± 0.25^{000}
Rotenone 5 μM	7.46 ± 0.98★★★	1.94 ± 0.25	13.84 ± 0.32★★★	3.64 ± 0.08^{000}
Oligomycin 1 μg/μl	7.67 ± 1.39★★★	1.99 ± 0.36	12.07 ± 1.14★★★	3.18 ± 0.30^{000}

[†]Chromaffin cells (10^6) were incubated for 4 hr (A) or 24 hr (B) in the absence (control) or presence of indicated ROS-producing compounds in DMEM containing 10% FCS. Afterwards, 50 μM hydroethidine was added and the ethidium fluorescence production measured as indicated in Materials and Methods. The slopes of the lines (a) fitting the fluorescence intensity changes (an index of the rate of superoxide production) were individually calculated and averaged. Data are the mean slope values ± SEM of four different experiments each performed in triplicate. Statistics compare the effect of every different agent vs. control at 4 hr or 24 hr of incubation at ★$P < 0.05$, ★★★$P < 0.001$ or the differences between the same condition comparing 24 hr to 4 hr at $^{000}P < 0.001$ (two-way ANOVA test for multiple variables).

shape, with membrane blebs, whereas small numbers were notably sunken. Nuclei of nonviable cells (red nuclei in Fig. 3B′–D′) showed chromatin condensation and, in numerous cases, nuclear fragmentation and apoptotic cell bodies. These images contrast with the morphology of control cells (blue cells in Figure 3A′), which displayed a typical polygonal to round shape, with no nuclear staining. Semiquantitative analysis of nonviable cells assessed by PI staining showed the following percentages of cell death: 10.1% ± 2.2% of dyed cells in nontreated cells (control) and 54.0% ± 10.0, 29.2% ± 5.1, 30.8% ± 4.6%, and 32.8% ± 6% of dyed cells in 1 mM SNP-, 10 μM LPS-, 10 nM TNFα-, and 50 UI/ml IFNγ-treated cells, respectively.

Because the morphological changes in cells treated with NO-generating compounds were highly suggestive of apoptosis, we performed a study of morphology of the entire cell population by confocal microscopy after staining of cell cultures with SYTO 13 and neutral red, to determine whether apoptosis was produced only in the chromaffin cell population. SYTO 13 stains DNA of all cell populations with a blue-green color, whereas neutral red (NR), which is avidly accumulated by chromaffin cells (Winckler, 1974; Brooks et al., 1992), selectively stains cytoplasm of chromaffin cells. Treatment with 1 mM SNP (Fig. 4B,C) caused the presence of rounded cells with a tendency to be detached, as well as the presence of fragmented nuclei and apoptotic cell bodies (Fig. 4, inset). These changes were induced after 8 hr of treatment (Fig. 4B), reaching maximal levels after 24 hr (Fig. 4C), when a great number of detached dead cells and a number of apoptotic-like cells could be observed. These morphological changes were observed mainly in the chromaffin cell population with red-stained cytoplasm. Endothelial cells, which appear in a lower plane with a certain refringence, were not red stained and died by necrosis in the very first hours of treatment. Treatment with 1 mM P induced more important morphological changes than treatment with SNP, these changes appearing after 6 hr of treatment (Fig. 4D). Cells suffering apoptosis were mainly chromaffin cells, which were rounded and had a tendency to be detached from well dishes. Endothelial cells appear below chromaffin cells, blue stained, with cell nuclei bigger than those of chromaffin cells and with broken connections. Cell death induced by P was time dependent, being maximal at 24 hr (Fig. 4F). At this

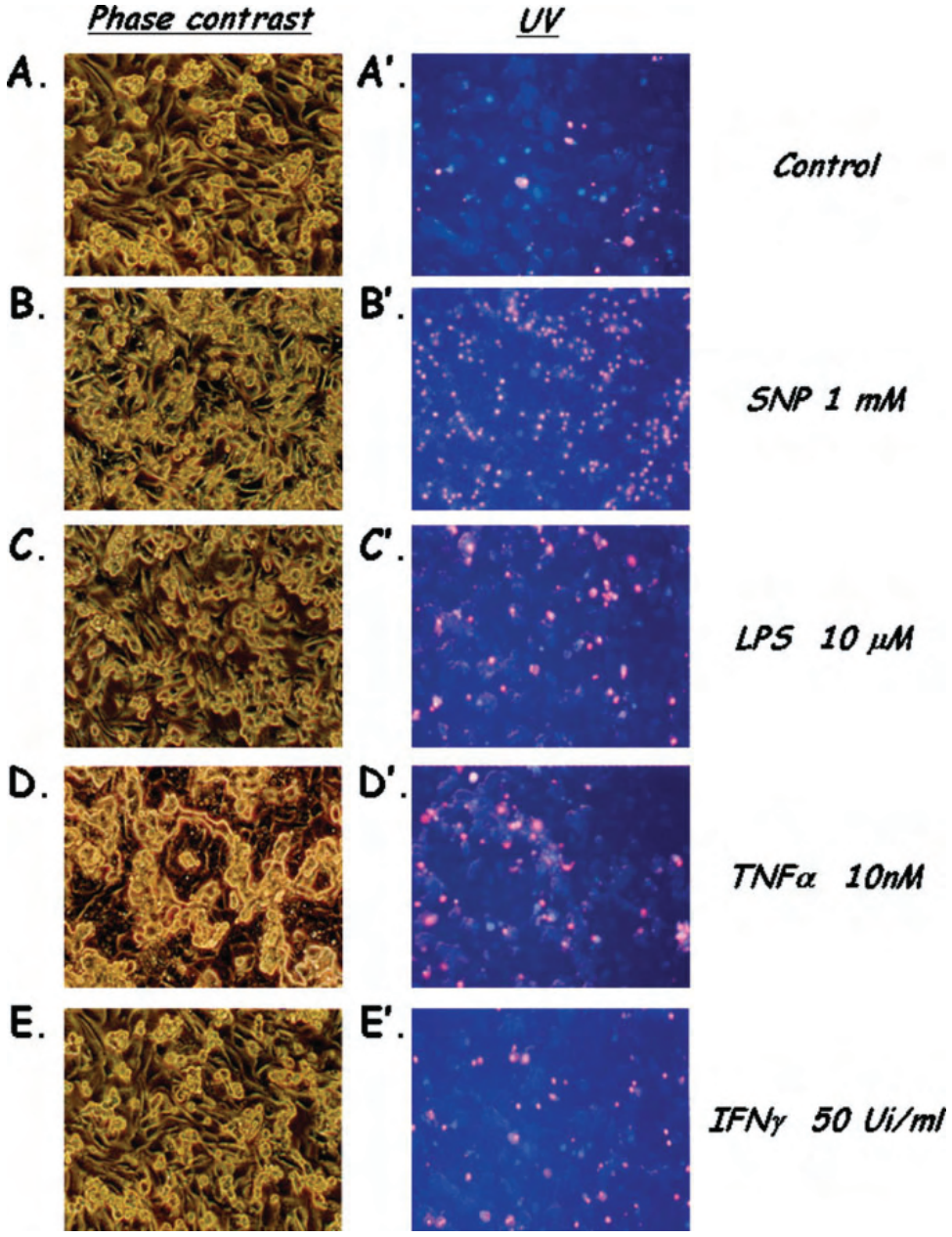

Fig. 3. Morphological analysis of nuclear chromatin in chromaffin cells stained with the DNA binding fluorochrome Hoechst 33342 and propidium iodide with a fluorescence microscope. In control cultures (**A**), viable cells show blue, round nuclei (Hoechst 33342 staining). Necrotic cells (propidium iodide, nuclei red) were present only in the cells with disrupted plasma membrane. Apoptotic cells were hardly observed in control cultures. In the presence of SNP (**B**) or cytokines (**C–E**), NO-induced necrosis in chromaffin cells after 24 hr of incubation was assessed by propidium iodide staining and apoptotic cells by the characteristic chromatin condensation and nuclear fragmentation of Hoechst 33342-stained cells and propidium iodide-stained cells.

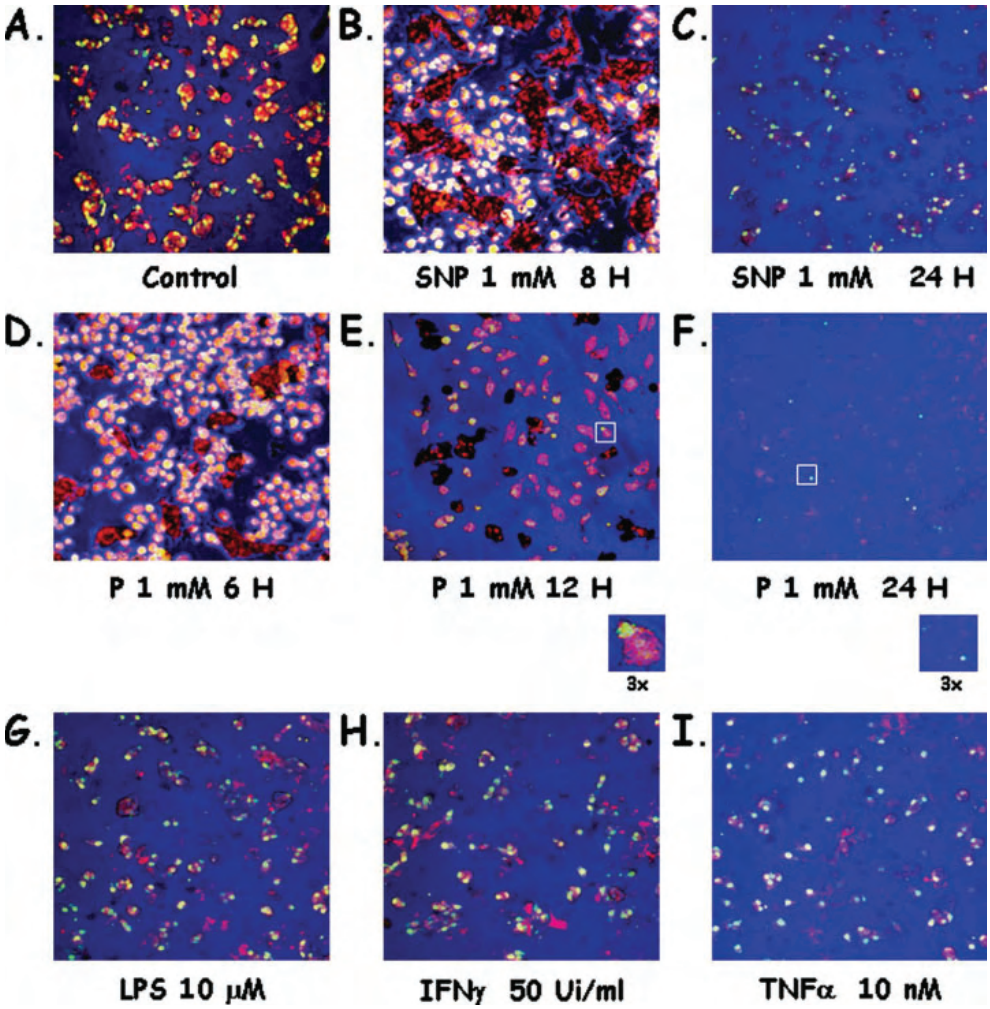

Fig. 4. Morphological analysis of nuclear chromatin in chromaffin cells stained with the DNA binding fluorochrome SYTO 13 and with the chromaffin cell cytosol fluorochrome neutral red via confocal microscopy. Chromaffin cells were treated for 24 hr with culture medium (control; **A**); 1 mM SNP for 8 and 24 hr (**B,C**); 1 mM P for 3, 12, and 24 hr (**D–F**); or LPS and cytokines for 24 hr at indicated doses (**G–I**). Cells were stained for 15 min with SYTO 13 and neutral red as described in Materials and Methods and then analyzed via confocal microscopy.

time, only a few apoptotic bodies and a high amount of cell debris appeared. In these cases, both chromaffin and endothelial cells displayed cellular death. However, analysis of morphological characteristics seems to show that, although chromaffin cells suffered mainly an apoptotic process, endothelial cells suffered mainly a necrotic death (Fig. 4F), although these experiments do not allow us to discount the possibility that some endothelial cells could suffer apoptosis. The fact that only a few cells appear after 24 hr of P treatment is due to prior washes for eliminating serum.

Finally, treatment of chromaffin cell cultures for 24 hr with LPS or cytokines only slightly modified chromaffin cell morphology, which shows numerous apoptotic bodies, the effects of TNFα being more powerful than those of the other two cytokines (Fig. 4G–I). These compounds do not seem to affect the contaminant endothelial cells. Taken together, these results indicate that both exogenously and endogenously generated P and NO induce characteristic morphological changes of cellular death in chromaffin cells, apoptotic features being predominant.

TABLE IV. Effect of SNP and Peroxynitrite on Apoptosis at Short and Long Incubation Times†

	% M1 (apoptosis)	
Conditions	A) 8 Hr	B) 24 Hr
Control	1.40 ± 0.14	2.86 ± 0.31^{000}
-FCS	1.90 ± 0.50 ns	$3.80 \pm 0.40^{\star 000}$
SNP 1 mM	1.10 ± 0.25 ns	$5.00 \pm 0.45^{\star\star 000}$
P 1 mM	$2.47 \pm 0.26^{\star}$	$16.80 \pm 2.03^{\star\star\star 000}$
Pd 1 mM	1.59 ± 0.05 ns	3.47 ± 0.66^{000}

†Chromaffin cells were stimulated for 8 hr (A) or 24 hr (B) with SNP, P (peroxynitnite), or Pd (deactivated peroxynitrite) in the presence of FCS. Results (mean ± SEM of three experiments, each performed in duplicate) were compared with apoptosis obtained in the absence of FCS (-FCS), and expressed as % M1 (apoptosis). Statistics compare the effect of different agents and control at 8 hr or 24 hr ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$) and the effect of time exposure for each condition ($^{000}P < 0.001$); (two-way ANOVA test).

NO Donors, Peroxynitrite, and Cytokines Increase the Number of Cells With Low DNA Content and Modify the Number of Cells in Different Phases of the Cell Cycle

For quantifying the NO- and P-induced apoptotic cell death in chromaffin cells, their DNA content was first analyzed by flow cytometry after staining with PI. SNP and P treatment induced a time-dependent increase in the percentage of cells with DNA content below 2C, which is not reproduced by Pd (Table IV). Peaks of 2 mM SNP-, SNAP-, and P-treated cells presenting DNA content below 2C after 24 hr are shown in Figure 5A. SNP, SNAP, and P at concentrations ranging from 10 μM to 2 mM induce a dose-dependent increase in apoptosis (Fig. 5B,C), the smaller effect corresponding to SNAP, followed by SNP (Fig. 5B) and then P (Fig. 5C; two, three, and seven times basal levels at 2 mM concentrations of SNAP, SNP, and P, respectively).

For studying the effect of these NO donors and P on the cell cycle, the flow cytometry analysis of cellular DNA stained with PI was also used and results compared with their effects on apoptosis. SNP and SNAP at concentrations ranging from 10 μM to 2 mM induce a small increase in the percentage of cells in G_0G_1 phase, which is accompanied by a parallel dose-dependent decrease in the percentage of cells in G_2M and S phases of cell cycle (Fig. 6A). By contrast, P induced statistically nonsignificant variations in G_0G_1 phase but induced an increase in the percentage of cells in both G_2M and S phases of cell cycle at concentrations ranging from 10 to 500 μM, followed by a decrease in the number of cells in both phases of the cell cycle (Fig. 6B). Changes induced by P were not mimicked by Pd which, as much the same as in apoptosis, did not produce any significant change in different phases of cell cycle.

With regard to the effects of LPS and cytokines on apoptosis and cell cycle, these compounds, alone or in combinations, induced, just like NO donors and P did, a time-dependent increase in the percentage of cells with a DNA content below 2C (Fig. 7A,B). This effect was inhibited by the NOS inhibitors L-NMA and thiocitrulline (Fig. 7B), compounds that do not produce apoptosis by themselves (Fig. 7B, inset). This increase in apoptosis was parallel to a decrease in the number of chromaffin cells in phase G_2M, as happens in the case of NO donors and P (Fig. 7C). However, in contrast to these compounds, cytokines did not induce any change in the number of cells in G_0G_1 phase and increased, not decreased, the number of cells in S phase (Figs. 7C).

For studying the specificity of changes induced in the cell cycle by NO-generating compounds and to confirm that these changes were specifically produced in chromaffin cells, we studied the ability of NGF (whose receptors are expressed only in chromaffin cells) to reverse these effects. We can see in Table V that changes in G_2M and S phases of the cell cycle induced by all these NO-generating compounds were reversed by NGF, which was able not only to protect chromaffin cells from NO-induced apoptosis but also to reverse the SNP and P induced decrease in percentage of cells in G_2M and S phases. NGF was also able to increase significantly the percentage of cells in G_2M phase decreased by cytokines and even to produce a significant increase in the number of cells in S phase not affected by cytokines and LPS (Table V). Insofar as NGF receptors are expressed only in chromaffin cells, these results seem to confirm that changes induced by NO on cell cycle are produced in chromaffin cells, ruling out the contribution of contaminant endothelial cells to the observed effect of NO on cell cycle.

NO Donors, Peroxynitrite, and Cytokines Induce DNA Fragmentation in Chromaffin Cells

For confirming that NO donors and P induce a process of apoptosis, some other biochemical methods were used, such as analysis of the presence of DNA fragments in the cytoplasmic fraction by agarose gel electrophoresis and measurement of caspase 3 activity. Study of the extranuclear DNA in cells untreated or treated with NO donors, P, or cytokines for 24 hr revealed an induction of DNA fragmentation by all these compounds, as shown in Figure 8. NO donors-, cytokines-, and P-treated cells presented different low-molecular-weight DNA fragments, resembling a "ladder" (not present in control cells), showing the cleavage of genomic DNA occurring in cells undergoing late apoptosis. The NO- and P-induced DNA fragmentation was specific, in that KFeCN (the vehicle of SNP) and Pd cause a very small effect on DNA cleavage.

Caspase 3 and 9 Activation and Cytochrome c Release Mediates NO-, Cytokine-, and P-Induced Apoptosis

For addressing whether caspases contribute to NO donors-, cytokines-, and P-induced apoptosis, chromaffin cells were incubated with SNP, LPS, cytokines, or P

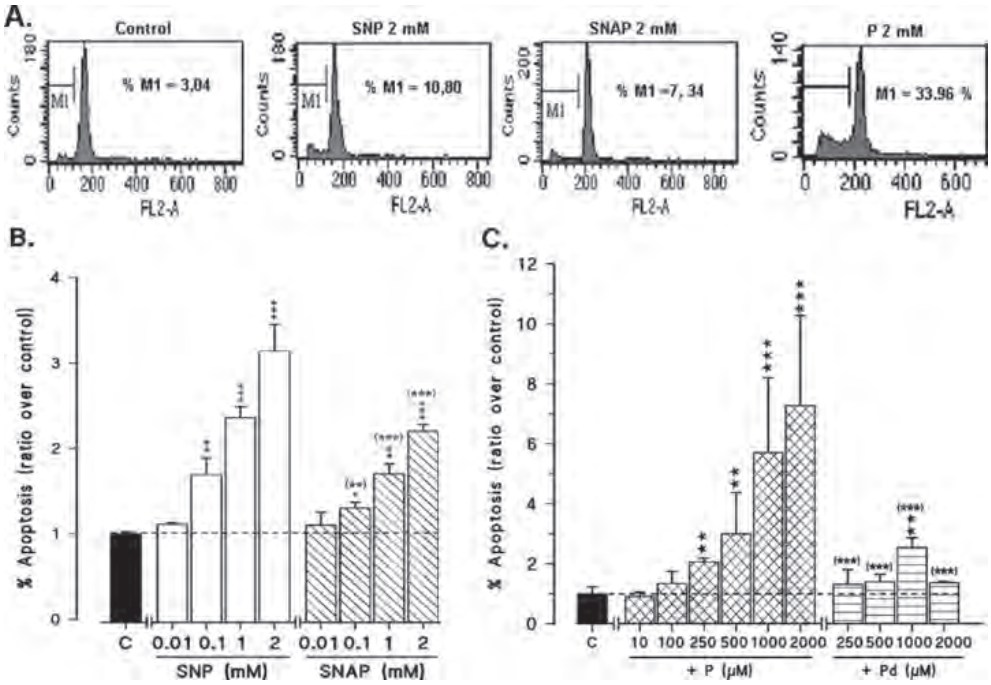

Fig. 5. NO donors and peroxynitrites induce a decrease in DNA content in chromaffin cells. **A:** DNA content histograms from flow cytometry measurements in bovine chromaffin cells untreated (control) or treated for 24 hr with 2 mM SNP, SNAP, or P carried out as described in Materials and Methods. M1 fractions represent the percentage of cells with DNA content below 2C (apoptotic cells). **B,C:** Dose-response curves showing apoptotic effect of NO donors (SNP and SNAP; B) or peroxynitrite (P and Pd; C). Percentage apoptosis was expressed as ratio over basal, and mean values ± SEM of three separate experiments each performed in duplicate are represented in each bar. Statistics compare the effect of SNP or P with the control values (vertical $^{**}P < 0.01$, $^{***}P < 0.001$) or in B- *differences between the same doses of SNP and SNAP or P and Pd (horizontal $^{\star}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$; two-way ANOVA).

for 24 hr in the presence or absence of Ac DEVD-CHO (CPP32), a cell-permeable compound that blocks caspase 3-like enzymes. DEVD-amc cleaving activity by caspase 3 was clearly increased after 24 hr of incubation with all these compounds (Fig. 9). The increase in caspase 3-like activity induced by all of these NO-generating compounds was almost completely reversed in the presence of 50 nM CPP32.

To determine whether caspase 3 and 9 were involved in NO-, cytokines-, and P-induced chromaffin cell apoptosis, we assayed the effect of CPP32 and Ac Z-LEHD-FMK (which block caspase 3- and caspase 9-like enzymes, respectively) on NO-induced apoptosis and inhibition of cell cycle. Both inhibitors, at 1 and 50 nM concentrations, produced a significant dose-dependent decrease in apoptosis induced by SNP, P, and cytokines such as IFNγ (Fig. 10A,B). Moreover, these compounds were also able to reverse the inhibition induced by all these NO-inducing compounds (especially the inhibition caused by SNP and cytokines) on the number of cells in G_2M phase, but they only slightly reversed the decrease in the percentage of cells in S phase in the case of P.

After viewing these results, we decided to study whether caspase activation induced by NO could be a consequence of cytochrome c release by mitochondria. After incubation of cells with NO-generating compounds, mitochondria were separated from cytosol, and cytochrome c release and content were analyzed by Western blot as described in Materials and Methods. As shown in Figure 11, cytochrome c release was significantly increased in chromaffin cells treated with SNP, P, LPS, and cytokines at 24 hr, the maximal effects being induced by SNP, P, and IFNγ. In contrast, levels of β-actin (control) remained unchanged (not shown).

DISCUSSION

NO is a diffusible messenger involved in several pathophysiological processes, including immune-mediated cytotoxicity and neural cell killing. NO or its redox products, such as P, can cause DNA damage and activate

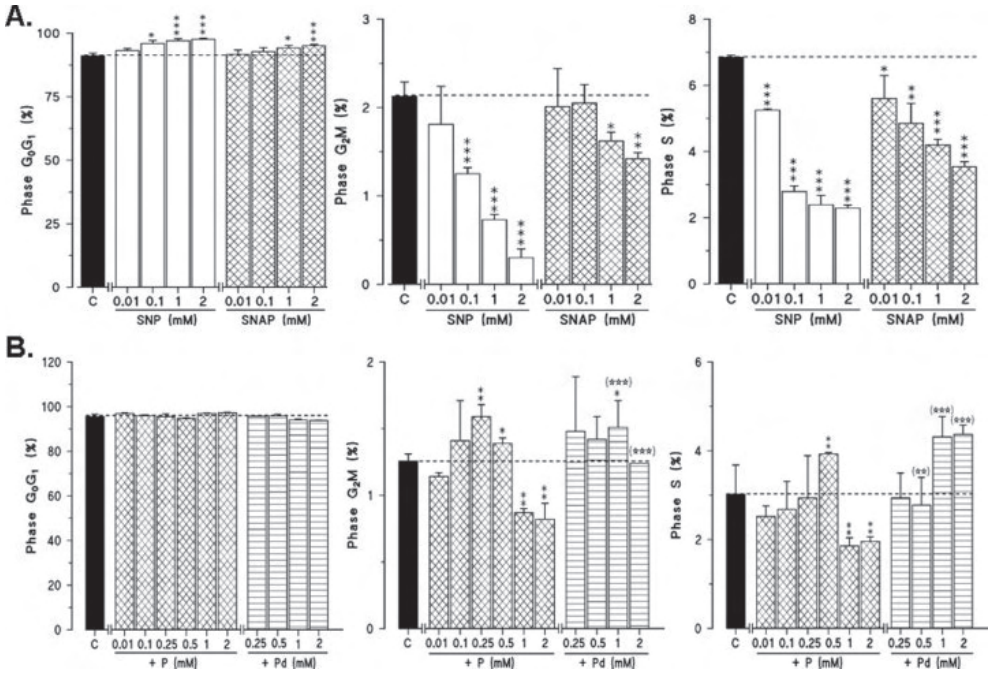

Fig. 6. NO donors and peroxynitrite induce a decrease in the number of chromaffin cells in G_2M and S phases of the cell cycle. Dose-response curves showing apoptotic effect of NO donors (SNP and SNAP; **A**) and peroxynitrite (P and Pd; **B**) on percentage of cells in different phases of the cell cycle. Results represents mean ± SEM of three determinations each performed in duplicated (n = 6). Statistics compare the effect of SNP or P with the control values ($^{**}P < 0.01$, $^{***}P < 0.001$) or the difference between the same doses of P and Pd ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; two-way ANOVA).

subsequent lethal reactions, including energy depletion and cell necrosis. NO induces apoptosis in several systems, regardless of its origin: endogenous production in response to cytokines or generation by chemical breakdown of donor molecules (Albina et al., 1993; Ankarcrona et al., 1994; Bal-Price and Brown, 2000; Figueroa et al., 2005). In this study, we demonstrate that in chromaffin cells NO and its oxidized derivate, P, are able to induce cellular death by a mixed necrotic and apoptotic mechanism, depending on time of stimulation and compound concentration.

Fluorescence and confocal microscopy analysis of morphological alterations in the presence of this compound showed three cell populations: completely blue-stained (live cells); broken cells with whole, red-stained nuclei (necrotic cells); and cells with blue-stained cytoplasm and red, fragmented nuclei (apoptotic bodies) typical of cells undergoing apoptosis. Specific staining of chromaffin cell cytoplasm with neutral red (Winckler, 1974; Brooks et al., 1992) helped us to see that, after 8 hr of treatment with NO donors, morphological apoptotic changes are produced mainly in chromaffin cells. Although P produces a mixed necrotic plus apoptotic cell death, apoptosis appearing earlier (from 3 hr incubations) and necrosis in a time-dependent way (being maximal at 24 hr of incubation), bacterial toxins and cytokines induce chromaffin cells to release NO, leading to cell death, mainly through an apoptotic mechanism. There are numerous examples in the literature demonstrating that NO and oxidized compounds are able to induce cell death by both necrotic and apoptotic mechanisms, depending on time of exposure and drug concentration. It has been postulated that mild stimuli produce mainly apoptosis, whereas strong stimuli produce necrosis over the long term and apoptosis over the short term (Bonfoco et al., 1995). For determining the quantity of NO stimuli required for inducing cell death, two important factors have to be taken into account: 1) the cellular content of antioxidant defenses and 2) the actual concentration of NO able to enter the cells. Chromaffin cells present high levels of GSH (about 5 nmol/5 × 10^6 cells; Jordan et al., 2002). We had previously seen that NO donors induced an increase in GSH levels, which could be related to the cells' attempt to protect themselves from the toxic NO stimulus. In contrast, P decreases GSH levels (unpublished results from our group). This result could be related to the strong death-inducing effect of this compound in chromaffin cells. In our

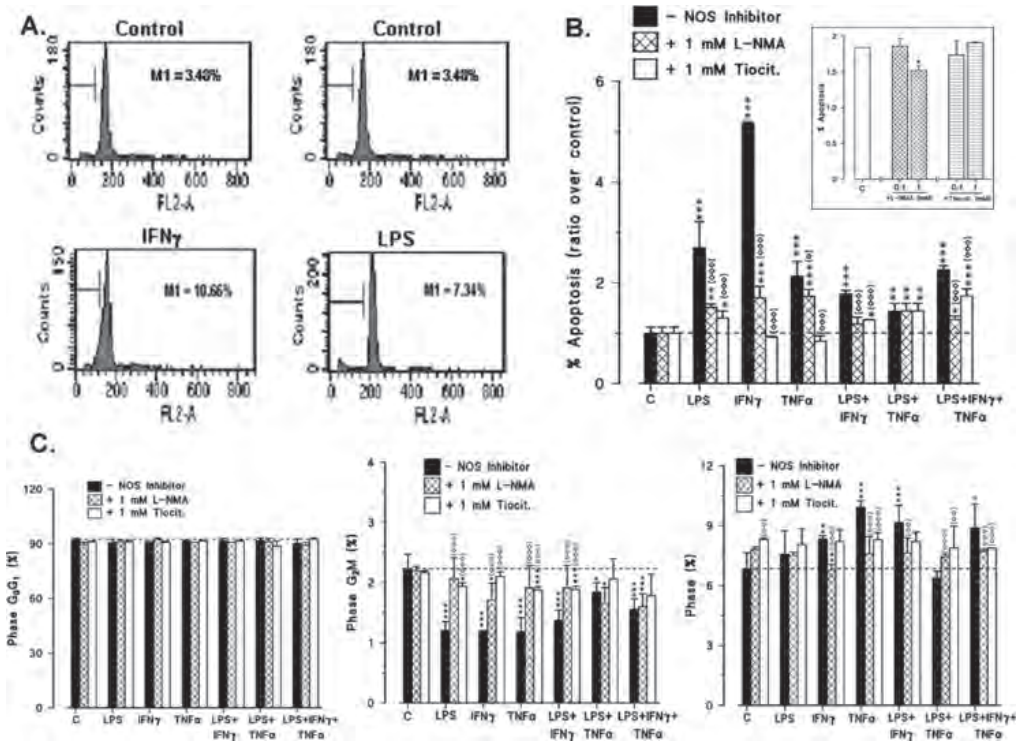

Fig. 7. Cytokines induce a decrease in DNA content specifically reversed by NOS inhibitors. Chromaffin cells were treated with 10 μM LPS, 50 UI/ml IFNγ, or 10 nM TNFα (alone or in specific combinations) for 24 hr, and DNA content and cell cycle were measured by flow cytometry. **A:** Representative records of DNA content histograms. M1 fractions represent the percentage of cells with DNA content below 2C (apoptotic cells). **B:** Quantification of apoptosis measures (mean ± SEM) from four experiments each performed by duplicate. Data in **inset** represent the basal effect of NOS inhibitors on apoptosis. **C:** Effect of cytokines (alone or in specific combinations) on different phases of the cell cycle in the absence or presence of NOS inhibitors L-NMA and S-methyl-L-thiocitrulline. Data are mean ± SEM from four experiments each performed in duplicate. Statistics compare the effect of cytokines with basal values at $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ and the effect of NOS inhibitors on the effect induced by each compound tested at $^{\diamond}P < 0.05$, $^{\diamond\diamond}P < 0.01$, $^{\diamond\diamond\diamond}P < 0.001$ (two-way ANOVA).

experiments, NO donors and P were used at high concentrations to ensure enough NO concentration inside the cells. It has been shown that PC12 cell stimulation with 1 mM P for 5 min produces submicromolar concentrations of this compound inside the cells during about 1 hr (Brunelli et al., 1995) and that millimolar concentrations of NO donors, such as SNP and SNAP, release small quantities of NO inside the cells for long periods of time (Bates et al., 1991), raising concentrations in the low micromolar range after cell stimulation (Ferrero et al., 1999). These NO levels are very similar to those produced by stimulation with cytokines, which stimulate NOS activity and expression in chromaffin cells or by activation of nNOS (2–4 μM) in conditions of brain ischemia (Malinski et al., 1993).

To analyze more deeply the type of NO-induced cell death in chromaffin cells, we studied the effect of these NO compounds on the release of LDH and ATP, nonspecific parameters that are altered in both necrotic and apoptotic cell death. At short incubation times (≤10 min), only P was able to increase LDH release, but, at long incubation times, all tested NO compounds increased the LDH release in a specific way, insofar as its effects were reversed by CPTio (an NO-trapping agent) in the case of NO donors and by NOS inhibitors in the case of cytokines. The effect of P, not reversed by CPTio at high concentrations of P, was partially reversed by NGF (90% at 40 ng/ml) and by glucose (50% at 50 μM concentration). These results seem to show the existence of a cell death with alterations in cell membrane permeability, which could be identified as a necrotic or late apoptotic cell death. This result was confirmed by measuring ATP release and ATP content. SNP was able both to increase ATP release and to decrease ATP intra-

TABLE V. Effect of NGF on Apoptosis and Changes in Cell Cycle Induced by NO-Generating Compounds in Chromaffin Cells[†]

Conditions	% M1	% G_0G_1	% G_2M	%S
NGF				
C	1.00 ± 0.12	1.00 ± 0.03	1.00 ± 0.20	1.00 ± 0.10
SNP 1 mM	1.97 ± 0.09★★★	1.03 ± 0.06	0.49 ± 0.09★★★	0.29 ± 0.02★★★
P 1 mM	6.47 ± 0.17★★★	0.87 ± 0.04★	0.33 ± 0.04★★★	0.34 ± 0.04★★★
Pd 1 mM	2.08 ± 0.20★★★	0.96 ± 0.02	0.45 ± 0.10★★★	1.11 ± 0.06
LPS	2.95 ± 0.56★★★	0.93 ± 0.05	0.61 ± 0.04★★	1.02 ± 0.09
IFNγ	1.66 ± 0.10★★★	0.97 ± 0.04	0.77 ± 0.02★	1.13 ± 0.11
TNFα	2.41 ± 0.29★★★	0.96 ± 0.06	0.66 ± 0.08★	0.96 ± 0.05
+NGF				
C	0.97 ± 0.09	0.93 ± 0.06	1.52 ± 0.15★★	1.70 ± 0.29★★★
SNP 1 mM	1.12 ± 0.06★^{000}	1.02 ± 0.03★	0.96 ± 0.10^{000}	0.69 ± 0.15★★^{000}
P 1 mM	2.67 ± 0.15★★★^{000}	0.96 ± 0.03^{0}	1.16 ± 0.05^{000}	0.74 ± 0.02★★^{000}
Pd 1 mM	1.25 ± 0.10★^{000}	1.01 ± 0.02	0.58 ± 0.10★★^{0}	0.83 ± 0.10★^{00}
LPS	1.05 ± 0.05^{000}	0.95 ± 0.02	1.45 ± 0.10★★^{000}	1.45 ± 0.19★★★^{00}
IFNγ	1.18 ± 0.10★^{000}	0.95 ± 0.01	1.72 ± 0.19★★★^{000}	1.33 ± 0.10★★^{0}
TNFα	1.07 ± 0.04^{000}	0.94 ± 0.02	1.34 ± 0.10★★^{000}	1.64 ± 0.24★★★^{000}

[†]Chromaffin cells were treated for 24 hr with indicated compounds in the absence or presence of 50 ng/ml NGF, and apoptosis and cells cycle were measured by flow cytometry as indicated in Materials and Methods. Results correspond to the mean ± SEM of three experiments each performed in duplicate. Statistical significances indicated by asterisks compare the effect of different compounds on apoptosis or cell cycle at ★$P < 0.05$, ★★$P < 0.01$, ★★★$P < 0.001$ and that indicated by circles compare the effect of NGF on changes in apoptosis or cell cycle induced for each compound at $^{0}P < 0.05$, $^{00}P < 0.01$, $^{000}P < 0.001$ (two-way ANOVA test for multiple variables). Control basal values were % M1 = 3.17 ± 0.40; % G0G1 = 88 ± 3; % G_2M = 1.37 ± 0.3 and % S = 7.60 ± 0.8.

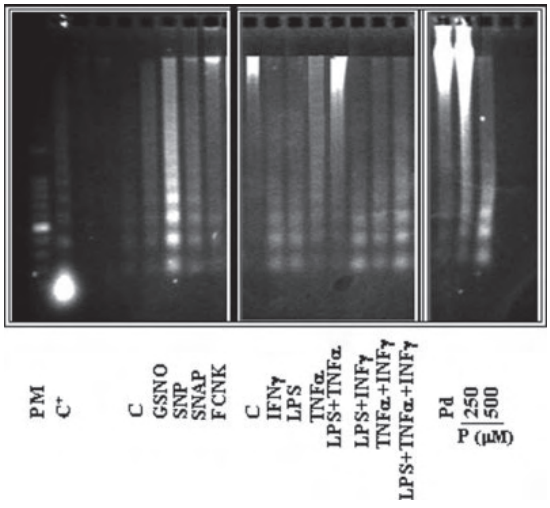

Fig. 8. Effects of NO donors, cytokines, and P on DNA fragmentation in bovine chromaffin cells. Representative agarose gels of DNA fragmentation. Chromaffin cell cultures were exposed to NO donors, cytokines, or P for 24 hr, and cytoplasmic DNA was extracted and analyzed as described in Materials and Methods. PM = molecular weight markers (100-bp ladder DNA); C^+ represents a positive control.

cellular content, effects that could be due to either an alteration in cell membrane permeability or a decrease in ATP synthesis. Given that, in SNP-treated cells, total ATP content (released ATP + intracellular ATP remaining content) is not significantly different from that in the control cells, it is likely that NO-induced ATP release could be due to an alteration in membrane permeability produced by a necrotic cell death or, as suggested by our recent results, that these effects are related to NO's ability to release cytosolic ATP by reversing the plasma membrane anion transporter (unpublished results). In this way, given that there is a 5:1 CAs:ATP ratio in chromaffin cells (Sillero et al., 1994) and that NO donors are not able to modify either CA secretion or its content (Vicente et al., 2005), the increase in ATP release must be cytosolic, not intragranular, and probably is related to its effect on specific anionic transporters. On the other hand, because the effect of NO donors on LDH and ATP release was reversed by both CPTio and SOD, it is plausible that the toxic effects of NO donors are due not only to NO but also to P. In fact, as shown in this paper, NO donors are able to increase the O_2^- production in chromaffin cells and, thus, to originate P.

Once determined that NO and its oxidative derivates are able to induce cell death in chromaffin cells, we tried to study NO-induced apoptosis in depth. Apoptosis was first assessed by flow cytometry, staining the cells with PI and measuring DNA content. All NO assayed compounds (cytokines and their combinations, NO donors, and P), were able to induce peaks of cells presenting a DNA content below 2C, which increase with dose and time of incubation with these NO com-

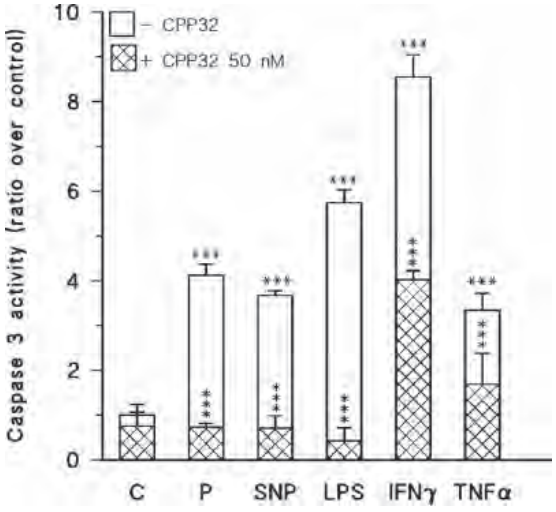

Fig. 9. NO induction of caspase 3 activity in bovine chromaffin cells and reversal of this effect by caspase 3-like activity specific inhibitor CPP32. Chromaffin cells were treated with NO donors (1 mM SNP, SNAP, and GSNO), 0.5 mM P, 10 μM LPS, 50 UI/ml INFγ, or 10 nM TNFα for 24 hr in the absence or presence of 50 nM CPP32. Caspase 3 activity was measured in cell lysates by using the fluorogenic substrate peptide DEVD-amc as indicated in Materials and Methods. Values represent the means ± SEM of at least three experiments each performed in duplicate and are expressed as a ratio over control (C = 7.20 ± 0.11 u.a.f./10^6 cells and CPP32 = 4.84 ± 0.1 u.a.f./10^6 cells). Statistical significances shown horizontally express the effect of different treatments on basal control (***$P < 0.001$), and statistical significances shown vertically represent the inhibition by CPP32 of caspase 3-like activity induced by the different treatments (***$P < 0.001$; multivariance analysis of ANOVA).

pounds. In the case of cytokines, the apoptotic effect was reversed in the presence of NOS inhibitors, indicating the participation of NO in the mechanism by which cytokines induce apoptosis. In the case of NO donors, the effect was dose-dependent and specifically reversed by CPTio and SOD, which means that, at least at high doses, P is also involved in the NO donor apoptotic effect. The effect of P was also specific, in that it was not mimicked by inactivated P. For confirming that these compounds induce an apoptotic process, we analyzed the presence of fragmented DNA in the cytoplasmic fraction by agarose gel electrophoresis, which reveals an induction of DNA fragmentation by all NO-assayed compounds. DNA from NO-treated cells presented different low-molecular-weight DNA fragments, resembling a ''ladder,'' that were not present in control cells, indicating the existence of apoptosis.

Activation of caspases is a final effector in the apoptotic cell death pathway. In chromaffin cells, NO donors were able to activate proteolytic caspases: caspase 9, the ''initiation'' caspase, and caspase 3, the ''effector'' caspase. Thus, on the one hand, all NO compounds tested were able to increase the activation of caspase 3, and, on the other hand, CPP32 and Ac Z-LEHD-FMK, specific inhibitors of caspase 3 and 9, respectively, were able to reverse NO-induced apoptosis. Because activated caspase 9 is involved in the activation of caspase 3, this being a key step in apoptosis, and cytochrome c release takes part, in turn, in caspase 9 activation, we studied the possible implication of cytochrome c release in NO-induced apoptosis. Results presented in this paper show that NO donors and cytokines were able to release cytochrome c from mitochondria to cytosol, showing the importance of mitochondrial involvement in the mechanism by which NO induces apoptotic death in chromaffin cells.

Results presented here demonstrate, for the first time, that NO is able to induce cell death in chromaffin cells by a mixed necrotic and apoptotic mechanism. There are very few studies described in the literature showing apoptotical death of chromaffin cells. Works by Jordan et al. (2000, 2002) show the effect of veratridine-induced apoptosis, mediated by superoxide anions, in chromaffin cells, and Ferrero and Torres (2001) show that YC-1, an NO-independent activator of soluble guanylyl cyclase that has a synergistic action with NO in stimulating cGMP synthesis, induces apoptosis in both endothelial and chromaffin adrenomedullary cells through a cGMP-independent mechanism. In our work, NO-induced apoptotic cell death in chromaffin cells is, at least in part, due to P formation and, possibly, carried out by S-nitrosylation or Tyr-nitration of specific proteins. The involvement of cGMP in this effect is very improbable, because, although in some experiments 8-Br cGMP was also able to induce apoptosis in a dose-dependent manner, neither ODQ, a specific inhibitor of soluble guanylate cyclase, nor KT5823, a specific PKG inhibitor, could reverse the effect of NO on apoptosis. Therefore, these results are in agreement with those of Ferrero and Torres (2001) on the apoptotic effect of YC-1 on chromaffin cells, which is independent of cGMP.

Our results on NO apoptotic effects in chromaffin cells are similar to those found by Desole et al. (1998), who showed apoptotic death induced by NO donors in PC12 cells, an effect in this case dependent of Fe mobilization by these cells, and are in agreement with those of Estévez et al. (1995) showing that these compounds induce cell death by two different mechanisms: necrosis, caused by P cell treatment with greater than 2 mM doses, and apoptosis, caused by treatment lasting for more than 4 hr. Because the cell cycle and apoptosis are two closely related events, we measured changes in the cell cycle parallel to measurements of apoptosis produced by NO compounds. In general, given the quiescent condition of chromaffin cells, changes in the cell cycle were very small, which is reflected by the high number of cells in the G_0G_1 phase. However, our results seem to indicate that apoptotic death induced by NO donors in chromaffin cells (increase in hypodiploid cell number) is

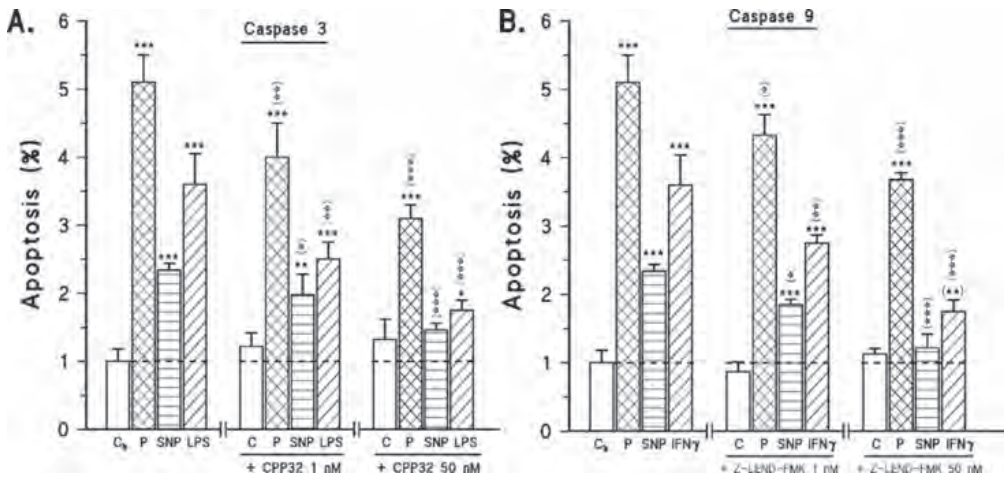

Fig. 10. Effect of caspase 3 (**A**) and caspase 9 (**B**) inhibition on apoptosis induced by different NO-increasing compounds. Chromaffin cells (10^6 cells/condition) were treated with the indicated compounds at concentrations indicated in Figure 8 for 24 hr in the absence or presence of CPP32 at 1 and 50 nM concentrations. Then, apoptosis was measured by flow cytometry as indicated in Materials and Methods. Values represent the means ± SEM of four experiments each performed in duplicate and are expressed as ratio over control. Statistical significances shown horizontally compare the effect of different treatments over the correspondent basal controls ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$), and statistical significances shown vertically represent the effect of CPP32 against the same treatment in the absence of this caspase 3 inhibitor ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; multivariance analysis of ANOVA).

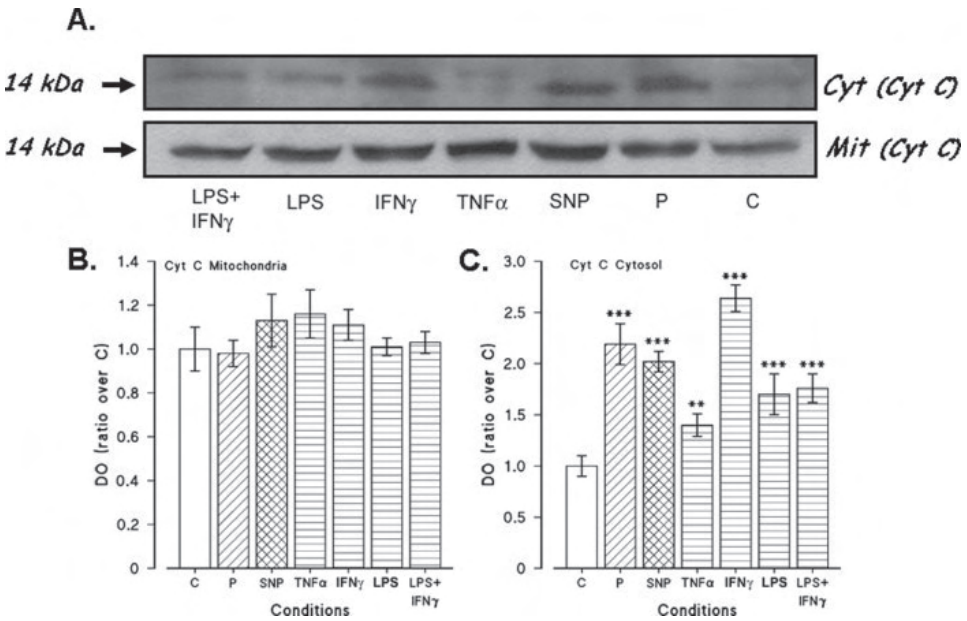

Fig. 11. Effect of NO-generating compounds on cytochrome c release into cytosol in chromaffin cells. Chromaffin cells (5×10^6 cells/condition) were treated for 24 hr with 0.5 mM P, 1 mM SNP, and 10 nM TNFα; 10 μM LPS; or 50 UI/ml IFNγ alone or at indicated combinations. Then, cells were lysed, and cytosolic (Cyt) and mitochondrial (Mit) fractions were separated and subjected to polyacrylamide gel electrophoresis and immunoblot analysis using an antibody specific for cytochrome c (Cyt C), as described in Materials and Methods. **A:** Representative gel of Western blot assay. **B,C:** Densitometric analysis of mitochondrial cytochrome c content (B) and cytocrome c released into cytosol (C). Data are means ± SEM from four different experiments. Statistical significances compare the effect of different treatments with the corresponding basal mitochondrial or cytosolic controls ($^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA).

concomitant with a cell cycle arrest in G_0G_1 phase and with a decrease in the number of chromaffin cells in G_2M and S phases, although, in the case of P, only high concentrations were able to induce changes in G_2M phase and, in the case of cytokines, we observed only a decrease in the number of chromaffin cells in G_2M phase.

Although effects of NO and P on the cell cycle have not been deeply investigated, there are numerous examples in the literature showing the inhibitory effect of NO on cell proliferation, mediated in many cases by cGMP. Thus, in prostatic cells, the apoptotic effect of NO as an inhibitor of cell proliferation seems to be due to cell cycle arrest in G1 phase, preventing the cells from entering into S phase (Guh et al., 1998). In vascular smooth muscle cells, apoptosis induced by NO is concomitant with a decrease in DNA synthesis mediated by the isoform Iα of PKG (Chiche et al., 1998). These results are in agreement with our results, although the participation of cGMP/PKG in these effects is very improbable, insofar as only high doses of the PKG inhibitor KT-5823 (5 μM), levels at which other kinases could also be inhibited, were able to reverse these effects partially. In fact, as shown by Ferrero and Torres, YC-1 also inhibits proliferation of chromaffin cells by a cGMP-independent mechanism. The induction of cell proliferation in cells derived from neural crest (such as sympathetic neurons, enterocytes, or chromaffin cells) by local neurogenic signals has been demonstrated not only in developing cells (Sweetser et al., 1997) but also in adult cells (Tischler et al., 1991). Thus, although adult chromaffin cells have been considered for many years mainly as postmitotic cells and completely undifferentiated, it has been shown that they can proliferate through life, being able even to induce hyperplasias and neoplasias, both spontaneously by an aging effect and after exposure to drugs such as reserpine, which produces, in short-term treatments, a CA depletion accompanied by an increase in cell proliferation, or with tumor-inducing agents in long-term treatments (Tischler et al., 1988). Although the underlying mechanisms of these effects are still unclear, neurogenic signals coming from preganglionic sympathetic cells, dorsal ganglia of spinal medulla, or other sources (including nitrergic signals) could be responsible for them. Another explanation for our results is that chromaffin cell cultures contain a small proportion of endothelial cells and that changes in cell cycle could be ascribed to these cells. The fact that caspase inhibitors are not completely able to block the effect of NO on the cell cycle but do block the effect of NO on apoptosis shows that the NO effects on these cells could be involved. However, results presented in this paper demonstrate that NO-induced changes in the cell cycle can be reversed by NGF, whose receptors are expressed only in chromaffin cells. In fact, NGF is able to induce an increase in G_2M and S phases under basal conditions by mechanisms involving the activation of both MAPKs and PI3K/PKB. This conclusion is supported by results from Lopez-Collazo et al. (1997) showing that exposure of adrenal cells from vascular endothelium to NO donors, proinflammatory cytokines, or LPS was unable to induce apoptosis in these cells. Therefore, our results seem to demonstrate that the effects on the cell cycle are produced in chromaffin cells and support the idea that these cells, with neuronal and endocrine properties, could be an interesting, simple model for elucidating the nature and regulation of neurogenic and transduction signals involved in neural cell cycle regulation.

In summary, all these results suggest that, in chromaffin cells, NO produces: 1) cellular death by necrosis, evidenced by LDH and ATP release, and 2) apoptosis, demonstrated by flow cytometry and DNA fragmentation. On the other hand, the caspases pathway and cytochrome c release are implicated in apoptosis, insofar as 1) NO donors, as well as cytokines (IFNγ and TNFα), are able to activate caspase 3 and cytochrome c release and 2) inhibitors of these enzymes reverse the NO-mediated apoptosis. Thus, these cells could be a good model for studying cell death from neurodegenerative diseases and neuroprotection. Given that NO plays an important role in apoptosis, the model of cell death and apoptosis by NO studied here could be very useful for elucidating the mechanisms by which catecholaminergic neurons die and the mechanisms by which neurotrophins and other neuroprotective agents may induce neuroprotection in important neurodegenerative diseases.

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Plasma Membrane and Vesicular Glutamate Transporter Expression in Chromaffin Cells of Bovine Adrenal Medulla

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The study of the functional expression of glutamate signaling molecules in peripheral tissues has received relatively little attention. However, evidence is increasing for a role of glutamate as an extracellular signal mediator in endocrine systems, in addition to having an excitatory amino acid neurotransmitter role in the CNS. Chromaffin cells are good models of catecholaminergic neurons, in which previous work from our group demonstrated the existence of both functional glutamate receptors and specific exocytotic and nonexocytotic glutamate release. In this work, the presence of specific plasma membrane (EAATs) and vesicular glutamate (VGLUTs) transporters has been investigated by using confocal microscopy, flow cytometric analysis, Western blot, and qRT-PCR techniques. We found specific expression of EAAT3, EAAT2, VGLUT1, and VGLUT3 in about 95%, 65%, 55%, and 25%, respectively, of the whole chromaffin cell population. However, chromaffin cells do not express VGLUT2 and have a very low expression of EAAT1. VGLUTs are localized mainly in the membrane fraction, and EAATs share their subcellular location between membrane and cytosolic fractions. Their estimated molecular weights were about 70 kDa for EAAT2, about 65 kDa for EAAT3, about 50 kDa for VGLUT1, and about 60 kDa for VGLUT3. RT-qPCR techniques confirm the expression of these glutamate transporters at the mRNA level and show a different regulation by cytokines and glucocorticoids between VGLUT1 and -3 and EAAT2 and -3 subfamilies. These interesting results support the participation of these glutamate transporters in the process of glutamate release in chromaffin cells and in the regulation of their neurosecretory function in adrenal medulla. © 2010 Wiley-Liss, Inc.

Key words: glutamate; excitatory amino acid transporters; glutamate release; cell death; chromaffin cells; adrenal medulla

The dicarboxylic amino acid glutamate is recognized as the major excitatory neurotransmitter in the central nervous system (CNS) of mammals, having important pathophysiological functions in synaptic plasticity and excitotoxicity (Platt, 2007). The role of glutamate as an excitatory neurotransmitter in mammals CNS has gained increasing support since the successful cloning of a great number of genes that encode the signaling machinery required for its neurocrine function in brain synapses. This machinery includes glutamate receptors as detection signals, plasma membrane glutamate transporters (excitatory amino acid transporters; EAATs) as finishing signals, and vesicular transporters (VGLUTs) as exocytotic releasing signals. EAATs are high-affinity Na^+-coupled glutamate transporters, which are members of the solute carrier family 1 (SLC1). There are five isoforms (EAAT1–EAAT5) of these transporters, both in neurons (EAAT3 or EAAC1 and EAAT4) and in astrocytes (EAAT1 or GLAST and EAAT2 or GLT1), EAAT5 being located in photoreceptor cells (for review see Kanai and Hediger, 2004). So far, three VGLUTs isoforms, which belong to the type I phosphate transporter family (also referred to as the *SLC17 family*), have been described (Hisano, 2003). The first characterized vesicular glutamate transporter, VGLUT1, is present in excitatory neurons of brain and cerebellum cortex, hippocampus, and thalamus; VGLUT2 in subcortical glutamatergic neurons between thalamus and spinal medulla; and the most recently cloned, VGLUT3, discretely distributed in brain, both in excitatory and in inhibitory neurons, as well as in cholinergic and monoaminergic

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neurons and glial cells (Takamori, 2006; Liguz-Lecznar and Skangiel-Kramska, 2007).

Until now, very little attention has been paid to the functional expression of glutamate transporters in peripheral nervous system and in nonneural tissues. Recent findings from molecular biology show new functions for glutamate as an extracellular signaling molecule in endocrine, autocrine, and/or paracrine tissues, such as pancreas, intestine, testis, placenta, kidney, and bone, as well as adrenal and pineal glands (Danbolt, 2001; Hinoi et al, 2004).

With regard to glutamate's function in chromaffin cells from adrenal medulla, neuroendocrine cells sharing a common embryologic origin with neurons and models of sympathetic postganglionic neurons, our research group demonstrated that this neurotransmitter may be involved in the regulation of catecholamine (CA) secretion, by interacting specifically with different glutamate receptor subtypes: NMDA, AMPA, KA, and t-ACDP (González et al., 1998; Arce et al., 2004). Thus, the role of glutamate as a potential neurosecretory regulator in this tissue has been established.

Several authors have suggested that adrenal glands receive sympathetic afferents and glutamatergic innervations along with their typical cholinergic innervations (Parker et al., 1993; Pyner and Coote, 1995). On top of this evidence, glutamatergic innervations to sympathetic adrenal neurons have also been found (Llewellyn-Smith et al., 1992, 1995). Moreover, our most recent work shows that chromaffin cells release glutamate, after stimulation with different secretagogues, by two processes, one exocytotic and Ca^{2+}-dependent and the other non-exocytotic, Ca^{2+}-independent, and probably mediated (as in neurons) by reversion of electrogenic transporters of plasma membrane (Romero et al, 2003). Thus, the question arises of whether specific plasma membrane and/or vesicular transporters are expressed in chromaffin cells and could mediate glutamate release by adrenal chromaffin cells.

The aim of this work was to study whether chromaffin cells express any vesicular and/or plasma membrane glutamate transporters and, thus, obtain additional evidence to support our previous results on the existence of both processes of glutamate release in chromaffin cells and a role for glutamate in the regulation of catecholamine secretion by chromaffin cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium, fetal calf serum, HEPES, and RNAse A were from Gibco BRL, U.K., and collagenase from *Clostridium histolyticum* (EC 3.4.4.19) was supplied by Boehringer Mannheim S.A. (Barcelona, Spain). Antibiotics, cytosine arabinoside, 8-fluoro-deoxyuridine (FDU), neutral red, and propidium iodide were from Sigma (Madrid, Spain). Amphotericin B was from ICN Ibérica S.A. (Barcelona, Spain). All other chemicals were reactive-grade products from Merck (Darmstadt, Germany). Rabbit anti-VGLUT1, anti-VGLUT2, and anti-VGLUT3 polyclonal antibodies were from Synaptic Systems (Göttingen, Germany); monoclonal mouse anti-EAAT3 antibody and polyclonal guinea pig anti-EAAT1 and anti-EAAT2 antibodies were from Chemicon (Temecula, CA). Mouse anti-β-actin, antisynaptophysin, and antityrosine hydroxylase monoclonal antibodies; rabbit antiglutamate decarboxylase 65/67 ($GAD_{65/67}$), antiphenyl ethanolamine-N-methyltransferase (PNMT) polyclonal antibodies, and anti-rabbit IgG TRITC, anti-mouse IgG FITC, or anti-guinea pig Cy3 or FITC conjugates secondary antibodies were from Sigma (St. Louis, MO). Other reagents were from Sigma or Boehringer (Mannhein, Germany).

Chromaffin Cell Primary Culture

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Pérez-Rodríguez et al. (2009). Cell viability and purity checking and cell plating in Petri dishes or on coverslips were performed as described elsewhere (Vicente et al., 2006), and cells were used 3–7 days after plating.

Synaptosomal Preparation From Cerebral Cortex

Synaptosomes from rat cerebral cortices were prepared and purified on discontinuous Percoll gradients (Amersham Biosciences, Uppsala, Sweden), as previously described (Millán et al., 2002).

Inmunocytochemistry and Confocal Microscopy

For immunocytochemical analysis, chromaffin cells or synaptosomes (used as positive controls) were plated on polylysine-coated glass coverslips. Cells (seeded at a density of 3×10^5/coverslip) were fixed for 2 min in ice-cold 1:1 acetone-methanol mixture (v/v) and washed twice with PBS. After blocking in PBS with 3% BSA/0.1% Tx-100 for 1 hr, the preparations were incubated with primary antibodies (rabbit anti-VGLUT1, anti-VGLUT2, and anti-VGLUT3 polyclonal 1:500; mouse anti-EAAT3 and antisynaptophysin monoclonal 1:500; and guinea pig anti-EAAT1 and anti-EAAT2 polyclonal 1:500) in PBS with 3% BSA for 1 hr at room temperature. After washing in PBS with 0.1% Tx-100 for 1 hr, secondary antibodies were added (FITC-labeled goat anti-mouse IgG, TRITC-labeled goat anti-rabbit IgG or Cy3-labeled goat anti-guinea pig IgG at 1:500 in PBS with 3% BSA) and incubated for another 1 hr. To study vesicular localization of VGLUTs or colocalization of plasma membrane and vesicular glutamate transporters, the cells were double immunostained with antibodies against one vesicular glutamate transporter and with antibodies against synaptophysin (monoclonal mouse antisynaptophysin), a marker of synaptic vesicles. The coverslips were mounted with Prolong Antifade in 50% glycerol in PBS containing 2.5% DABCO, and digital images were taken with a Leica confocal microscope and analyzed with Leica confocal software. Negative controls were made by incubating chromaffin cells or synaptosomes with secondary antibodies in the absence of primary antibodies. Thus, nonspecific signals could be detected.

Immunocytochemical Flow Cytometry Analysis

The quantification of glutamate transporter expression in chromaffin cells was performed in a Becton Dickinson FACScan Flow Cytometer (Becton-Dickinson, San Jose, CA). Basal or stimulated chromaffin cells (10^6/condition) were washed twice with PBS, harvested with trypsin-EDTA (0.25% trypsin, 1 mM EDTA), and pelleted by centrifugation at 2,000*g* for 5 min. After washing with ice-cold PBS, cells were fixed with 1 ml of 70% (v/v) methanol at –20°C for 2 min, centrifuged, and washed again with PBS. Immunostaining was performed by incubating cells with primary antibodies (1/100) in PBS with 3% BSA, for 1 hr at 37°C. After washing in PBS, secondary antibodies were added (FITC-labeled goat anti-mouse or anti-guinea pig or TRITC-labeled goat anti-rabbit IgGs) at 1:200 in PBS with 3% BSA and incubated for another 1 hr. Cells were finally centrifuged, washed, resuspended in cold PBS, and analyzed by flow cytometry.

Subcellular Fractionation of Chromaffin Cells: Cytosolic and Membrane Extract Preparations

Chromaffin cells (5×10^6), washed twice with cold PBS, were resuspended in a lysis buffer containing 250 mM sucrose, 25 mM Tris/HCl, pH 6.8, 1 mM EDTA, 0.05% digitonin, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin. Samples were centrifuged at 13,000*g* for 3 min at 4°C. Supernatants were taken and considered as cytosolic fractions. The pellets (containing the membrane fractions) were extracted with 40 mM HEPES, pH 7.6, 0.5 M EDTA, 1 M KCl, 5% glycerol, 0.2% Tx-100, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin and centrifuged at 13,000*g* for 3 min at 4°C. Supernatant was taken and considered as membrane fraction. Proteins in both cytosolic and membrane fractions were measured with the Bio-Rad (Hercules, CA) protein reagent.

Western Blot Analysis

Proteins from cytosolic and membrane factions were boiled in Laemmli sample buffer (200 mM Tris-HCl, 10% glycerol, 6% SDS, 4% β-mercaptoethanol, 2 mM EDTA, 0.02% bromophenol blue, pH 6.8), and equal amounts of protein (25–75 μg) were size-fractionated in a 10% acrylamide gel; transferred to a PVDF membrane; and, after blocking with 5% nonfat dry milk, incubated with the corresponding primary antibodies and visualized according to the ECL Western Blotting detection system (Amersham) as described by Vicente et al. (2002). Different exposure times were performed with each blot to ensure the linearity of the band intensities. Band intensities were measured on a densitometric scanner and normalized with respect to β-actin expression.

RT-PCR Analysis

An RNeasy Mini Kit was used for total RNA isolation. Reverse transcription (RT) was carried out for 1 hr at 55°C with oligodeoxythymidylate primer using 5 μg total RNA from each sample for complementary DNA synthesis. Semiquantitative and real-time quantitative PCR was performed in order to determine the levels of EAATs and VGLUTs and housekeeping GAPDH mRNAs by using the specific primers listed in Table I synthesized by Sigma-Aldrich.

TABLE I. Specific Primers

Primer	Sequence
GA3PDH forward	5′-CACAGTCAAGGCAGAGAACG-3′
GA3PDH reverse	5′-TACTCAGCACCAGCATCACC-3
EAAT1 forward	5′-GGTCACTGCAGTCATTGTGG-3′
EAAT1 reverse	5′-CACCAGCATCTGTAGCATCC-3′
EAAT2 forward	5′-TGCTGGACAGAATGAGAACG-3′
EAAT2 reverse	5′-TCGGTGCTGAGAGTCAATGG-3′
EAAT3 forward	5′-GGAGAAGCTCTCCAAGAAGG-3′
EAAT3 reverse	5′-CTCATTGTCAAGTGCTGTGG-3′
VGLUT1 forward	5′-TGGCCTCATACACGGTTCC-3′
VGLUT1 reverse	5′-GTGGAGGTAGCCACAATAGC-3′
VGLUT2 forward	5′-TTAGCTGGCATTCTTGTGC-3′
VGLUT2 reverse	5′-CAGGACTCTCGTAGGACACC-3′
VGLUT3 forward	5′-CTGCGTCATGTGTGTCAGG-3′
VGLUT3 reverse	5′-GGAGGTTGTAGCCAGTCTGC-3′

Real-time PCR. The SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the 7900 HT Fast Real-Time PCR system (Applied Biosystems) were used for detecting the real-time qPCR products of reverse-transcribed cDNA samples, according to the manufacturer's instructions. qPCR conditions were 95°C (10 min) followed by 40 cycles of 15 sec at 95°C and annealing for 1 minute at 60°C. Three independent quantitative PCR assays were performed for each gene and measured in triplicate. Three non-template controls (NTCs) were run for each qPCR assay, and genomic DNA contamination of total RNA was controlled using RT minus controls (samples without the reverse transcriptase).

Semiquantitative PCR. Conventional PCR amplifications were conducted in a 25 μl solution containing 1× PCR buffer, 0.2 mM dNTP mix, 1.5 mM magnesium chloride, 400 nM of each primer, 1 U DNA polymerase, and 2 μl cDNA template, corresponding to 5 μg total RNA in a 20 μl final volume. Negative control of amplification was performed with 2 μl water instead of cDNA template. Amplification conditions were 2 min at 95°C, 11 cycles of 30 sec at 95°C, 30 sec at 61°C, decreasing 0.5°C every cycle, and 20 sec at 72°C, followed by 23 cycles of 30 sec at 95°C, 30 sec at 55.5°C, and 20 sec at 72°C, and a final extension of 2 min at 72°C. Reactions were carried out in a thermal cycler. Ten microliters of the PCR products was resuspended in 6× loading buffer (30% glycerol, 0.5 μg/ml BrEt) and electrophoresed through 1.5% agarose in 0.5× TBE buffer (45 mM Tris-borate; 1 mM Na_2EDTA, pH 8.0) with 0.5 μg/ml BrEt for 1.5 hr.

Statistical Analysis

Data were expressed as mean ± SEM values of three or four independent experiments with different cell batches, each performed in duplicate or triplicate. Statistical comparisons were assessed by using one-way ANOVA (Scheffe's F test), followed in some instance by a two-way ANOVA test. Differences were accepted as significant at $P \leq 0.05$.

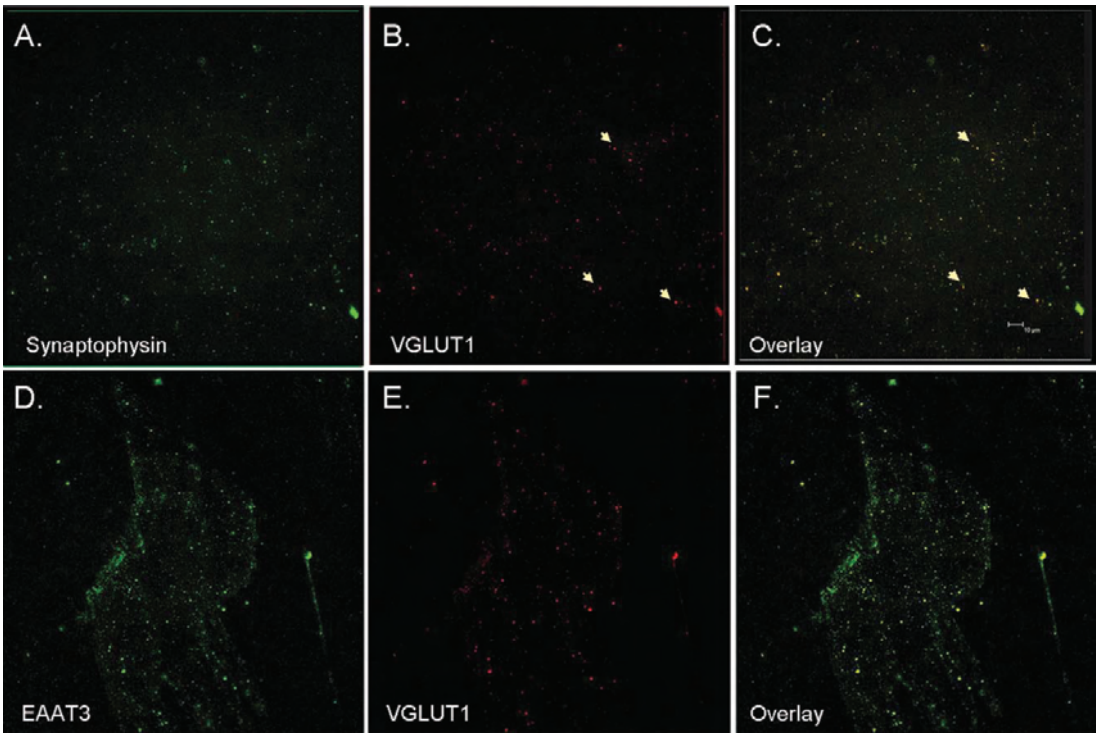

Fig. 1. Expression of VGLUT1 and EAAT3 in cerebral rat cortical synaptosomes determined by immunocytochemistry and confocal microscopy. Synaptosomes were fixed on polylysine-coated coverslips, and confocal images of double-stained nerve terminals were obtained by performing immunocytochemistry using antibodies against the vesicular glutamate transporter VGLUT1 (**B,E**) and the plasma membrane transporter EAAT3 (**D**). Specific primary antibody against synaptophysin was used to label synaptic vesicles (**A**). Nerve terminals were visualized with FITC filters (green) for synaptophysin (A) and EAAT3 (D) and with TRITC filters (red) for VGLUT1 (B,E) in a multispectral confocal microscopy Leica TCS-SP2-AOBS with a ×40 objective. Merged panels are shown in **C,F**. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS

Immunocytochemical Studies and Confocal Microscopy Reveal the Expression of Different Plasma Membrane (EAATs) and Vesicular (VGLUTs) Glutamate Transporters in Chromaffin Cells

To ensure the specific microscopic detection of fluorescent signals for glutamate transporters in chromaffin cells, previous immunocytochemical staining experiments for VGLUT1 and EAAT3 were performed in synaptosomes. In these experiments, a lower concentration of nerve terminals was attached to the polylysine-coated coverslips to facilitate visualization as described by Millan et al. (2002). Synaptosomes were labeled with antiserum against the vesicular protein synaptophysin, and glutamatergic nerve terminals were identified with antisera against the vesicular glutamate transporter VGLUT1 and the neuronal plasma membrane transporter EAAT3 (Fig. 1). Among the synaptophysin-containing particles (521 particles from three fields), VGLUT1 was detected in 59.8% ± 2.7% (mean ± SEM) of the terminals (Fig. 1A–C), a proportion very similar to that detected by Millan et al. (2002). Among the EAAT3-immunostained synaptosomes (582 particles from three fields) VGLUT1 was detected in 38.9% ± 2.4% (mean ± SEM) of the terminals (Fig. 1D–F).

Once we had ensured the accuracy of our immunochemical staining, we performed the same immunocytochemistry experiments in chromaffin cells, beginning with VGLUT1 and EAAT3 expression, given their known specific expression in neurons, and taking into account the neural origin of chromaffin cells. We used synaptophysin immunolabelling to ensure specific staining of the chromaffin cells. Results in Figure 2 show that, as in the case of synaptosomes, there is a specific immunolabelling for VGLUT1 in about 60–70% of chromaffin cells stained with synaptophysin (Fig. 2A–C), whereas specific immunostaining for EAAT3 was shown in almost the whole population of chromaffin cells (Fig. 2D). Figure 2E,F shows that the great majority of EAAT3-immunollabelled chromaffin cells were also specifically stained for VGLUT1, although with differences

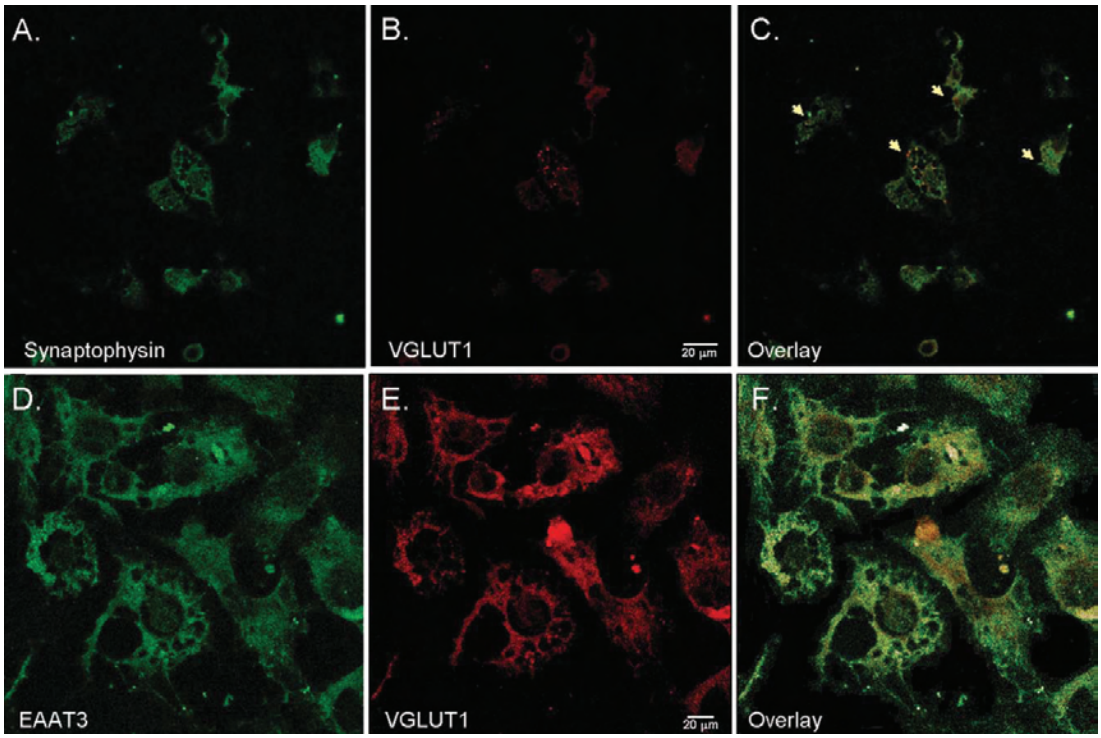

Fig. 2. Expression of VGLUT1 and EAAT3 in bovine chromaffin cells by immunofluorescence confocal microscopy. Chromaffin cells (3×10^5/coverslip) were fixed on polylysine-coated coverslips, and confocal images of double-stained cells were obtained by immunocytochemistry using antibodies against the vesicular glutamate transporter VGLUT1 (red; **B,E**) and the plasma membrane transporter EAAT3 (green; **D**). Antibody against synaptophysin was used to label secretory vesicles (green; **A**). Cells were visualized with FITC filters for synaptophysin (A) and EAAT3 (D) and with TRITC filters for VGLUT1 (B,E) in a multispectral confocal microscopy Leica TCS-SP2-AOBS with a ×40 objective. Merged panels are shown in **C,F**. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regarding the intensity of immunollabeling in different cells. These results suggest the colocalization of both neuronal glutamate transporters in the same population of chromaffin cells. To determine whether other vesicular or plasma membrane glutamate transporters were expressed in chromaffin cells, additional immunocytochemical experiments were performed for VGLUT2 and VGLUT3 as well as for EAAT1 and EAAT2. Results in Figure 3 indicate that, although no specific immunoreactivity was observed in the case of VGLUT2 (Fig. 3A–C), there was a specific immunolabelling for VGLUT3 in about 20–30% of chromaffin cells (Fig. 3D–F); however, specific immunolabelling for VGLUT3 was observed not only in chromaffin (i.e., cell 1) but also in cells not immunolabelled for synaptophysin (i.e., cell 2), which indicates that cells other than chromaffin could also express VGLUT3. In addition, a very weak immunolabelling was observed for EAAT1 (Fig. 3G–I) in a small number of chromaffin cells, whereas immunoreactivity for EAAT2 was stronger and was observed in the great majority of chromaffin cell population immunolabeled with synaptophysin (Fig. 3J–L). Thus, taken together, these results seem to indicate that chromaffin cells preferably express VGLUT1 and EAAT3, followed by VGLUT3 and EAAT2.

Quantification of the VGLUT and EAAT Expression in Chromaffin Cells by Flow Cytometry Techniques

To quantify, in a more accurate way, the cell subpopulation that expresses each glutamate transporter, we carried out quantitative studies by flow cytometry. To ensure the accuracy of the technique used for labeling specific chromaffin cell phenotypes, we examined the number of total catecholaminergic, adrenergic, and GABAergic chromaffin cells by using specific antibodies against tyrosine hydroxylase (TH), the limiting enzyme in catecholamine biosynthesis; phenyl ethanolamine N-methyl transferase (PNMT), the specific marker for adrenergic cells; and glutamate descarboxylase (GAD), the limiting enzyme for GABA biosynthesis. Results indicate proportions of 95.6% ± 2.8%, 75.9% ± 1.7%, and

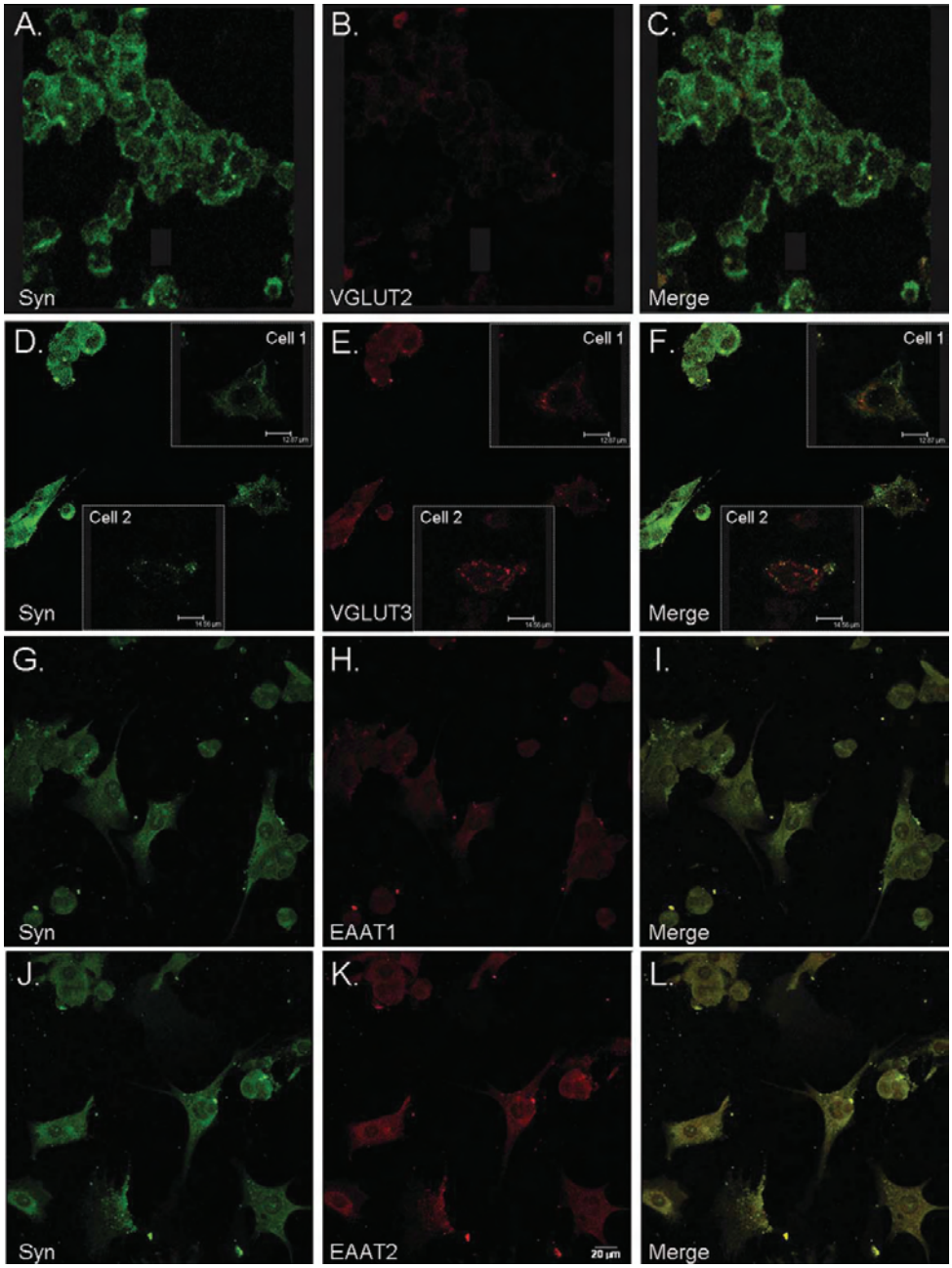

Fig. 3. Expression of VGLUT2 and -3 and EAAT1 and -2 in bovine chromaffin cells determined by immunofluorescence confocal microscopy. Expression of VGLUT2 (B) and -3 (E) and EAAT1 (H) and -2 (K) was examined by confocal microscopy as indicated in Figure 2. Double immnolabeling for each of these GLTs (red) and synaptophysin (green) indicated the absence of expression of VGLUT2 (**A–C**), the expression of VGLUT3 (**D–F**), the poor immunolabeling for EAAT1 (**G–I**), and the prominent immunolabeling for EAAT2 (**J–L**) in secretory vesicles immunolabeled for synaptophysin (D,E). Merged panels are shown in C,F,I,L. In F, cell 1 and cell 2 are examples of cells in which VGLUT3 signals colocalize (cell 1) or not (cell 2) with synaptophysin signals. Images are representative of five or six fields analyzed in three experiments performed with different cell batches. Scale bar = 20 µm.

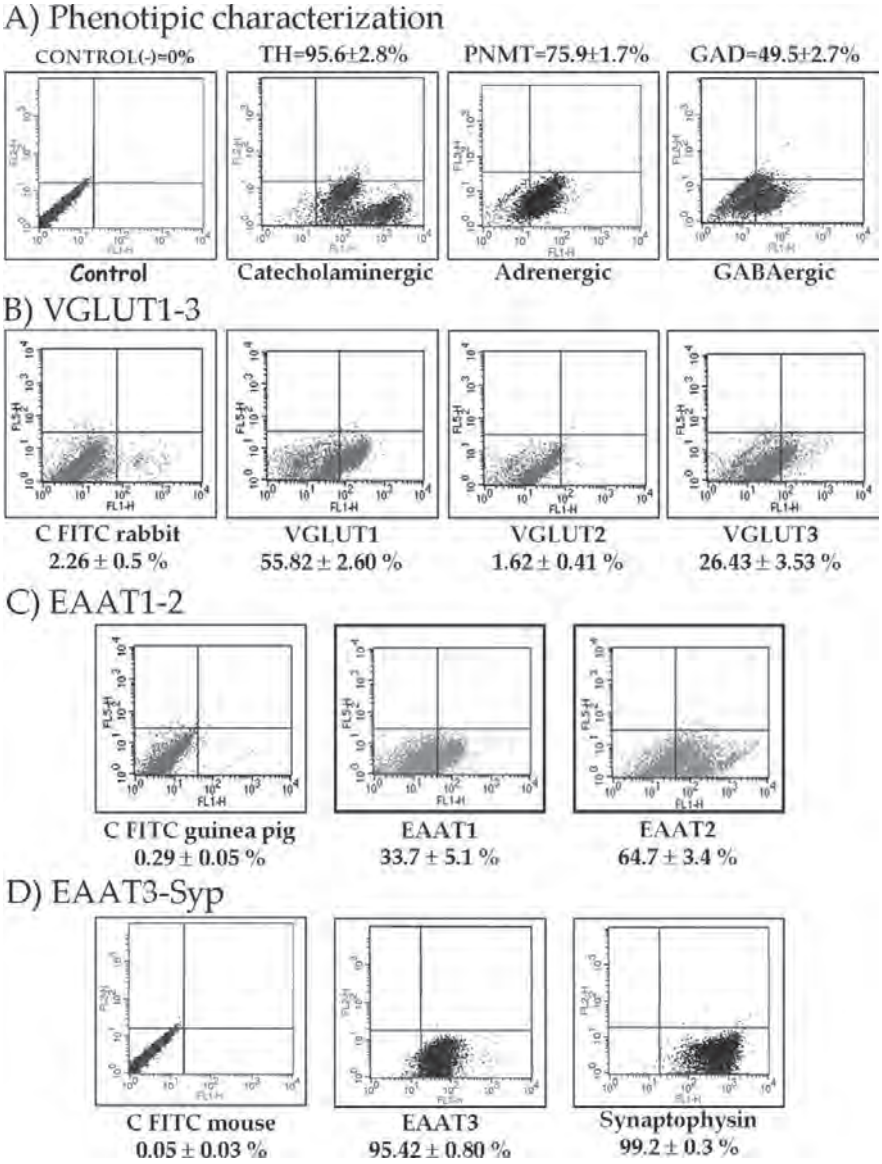

Fig. 4. Expression of glutamate transporters in chromaffin cells assessed by immunocytochemical flow cytometry techniques. Cells were harvested with trypsin-EDTA, pelleted, and fixed. Immunostaining was performed by incubation with primary antibodies against GLTs and secondary antibodies (FITC-labeled goat anti-rabbit for VGLUTs, anti-guinea pig for EAAT1-2, and anti-mouse for EAAT3 IgGs) and analyzed by flow cytometry as indicated in Materials and Methods. Figures represent plots of log fluorescence vs. cell count from flow cytometry. **A**: Phenotypic characterization of chromaffin cells by flow cytometry by using mouse anti-TH and anti-rabbit PNMT and anti-GAD antibodies. **B**: VGLUTs expression in different proportions by chromaffin cells. **C**: EAAT1 and EAAT2 expression in different proportions by chromaffin cells. **D**: EAAT3 expression in about the whole chromaffin cell population. Data indicate the percentage of expression (mean ± SEM) for different GLTs obtained from three separate experiments, from different cell batches, each performed in triplicate.

49.5% ± 2.7% of catecholaminergic, adrenergic, and GABAergic cells, respectively (Fig. 4A), proportions that are in the rank order of those observed in our previous studies (Oset-Gasque et al., 1998; Castro et al., 2003). Flow cytometry results of chromaffin cells immunolabelled with anti-VGLU1-3 transporters show that

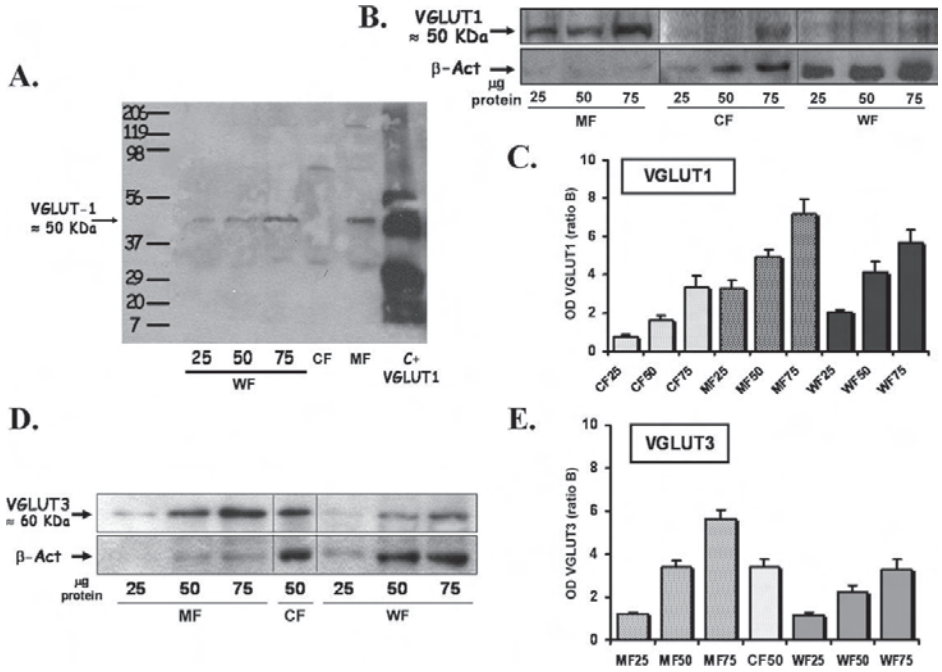

Fig. 5. VGLUT1 and VGLUT3 expression in subcellular fractions of bovine chromaffin cells determined by Western blot analysis. **A,B,D**: Representative images of Western blot experiments for VGLUT1 (A,B) and VGLUT3 (D) in whole fraction (WF), membrane fraction (MF), and cytosolic fraction (CF) of chromaffin cells at indicated amounts of total protein. Estimated molecular weights are indicated. **C,E**: Quantitative analysis of VGLUT1 (C) and VGLUT3 (E) expression in subcellular fractions of chromaffin cells. The data represent the ratios of densitometric values normalized against the ECL backgrounds obtained for each Western blot and are mean ± SEM values obtained from three experiments with different cell batches each performed ion duplicate. β-Actin signals were obtained in order to assess the purity of different fractions and the quantity of proteins in each assay.

55.82% ± 2.60% of cells express VGLUT1 and 26.43% ± 3.53% of cells express VGLUT3. However, only 1.62% ± 0.41% of cells were immunolabelled for VGLUT2 (Fig. 4B). These results confirm that chromaffin cells express VGLUT1 more abundantly, followed by VGLUT3, but that these cells do not seem to express VGLUT2. Similarly, we observed that while almost the whole population of chromaffin cells express EAAT3 (95.42% ± 1.80%; Fig. 4D; a proportion very similar to that observed for synaptophysin; 99.2% ± 0.83%); only 64.7% ± 3.4% of cells were immunolabelled for EAAT2, and only 33.7% ± 5.1% of cells were immunolabelled for EAAT1 (Fig. 4C). Thus, these results seem to confirm those of microscopic studies, described above, showing that the plasma membrane glutamate transporter expressed more abundantly in chromaffin cells is EAAT3, followed by EAAT2 and EAAT1.

Subcellular Localization of Glutamate Transporters Expressed in Chromaffin Cells Through Western Blot Techniques

The expression of VGLUT1 and -3 and EAAT3 and -2, shown above led us to perform a subcellular distribution study of these transporters in whole homogenates and cytosolic and membrane fractions of chromaffin cells by Western blot to determine the approximate molecular weight and have additional evidence on the localization of these transporters within these cells. Western blot analysis revealed the appearance of a band of approximately of 50 kDa mw, corresponding to VGLUT1, in whole homogenate fractions of chromaffin cells, the intensity of which was directly proportional to the protein amount in the sample (Fig. 5A). This signal was about three times stronger in membrane fractions than in cytosolic fractions ($P < 0.001$; ANOVA test) and was always proportional to the quantity of protein used for the assays (Fig. 5B,C), thus indicating that VGLUT1 is expressed mainly in particulate fractions of chromaffin cells.

We also found a band with an mw of about 60 kDa for VGLUT3 (Fig. 5D). In contrast to VGLUT1, the intensity of signals obtained for VGLUT3 expression was very similar in membrane and in cytosolic fractions, indicating a similar distribution of this vesicular transporter between the two subcellular compartments (Fig. 5E).

With regard to the EAATs expression, our results showed bands of expression for the three transporters at 62, 69, and 63 kDa mw for EAAT1, EAAT2, and

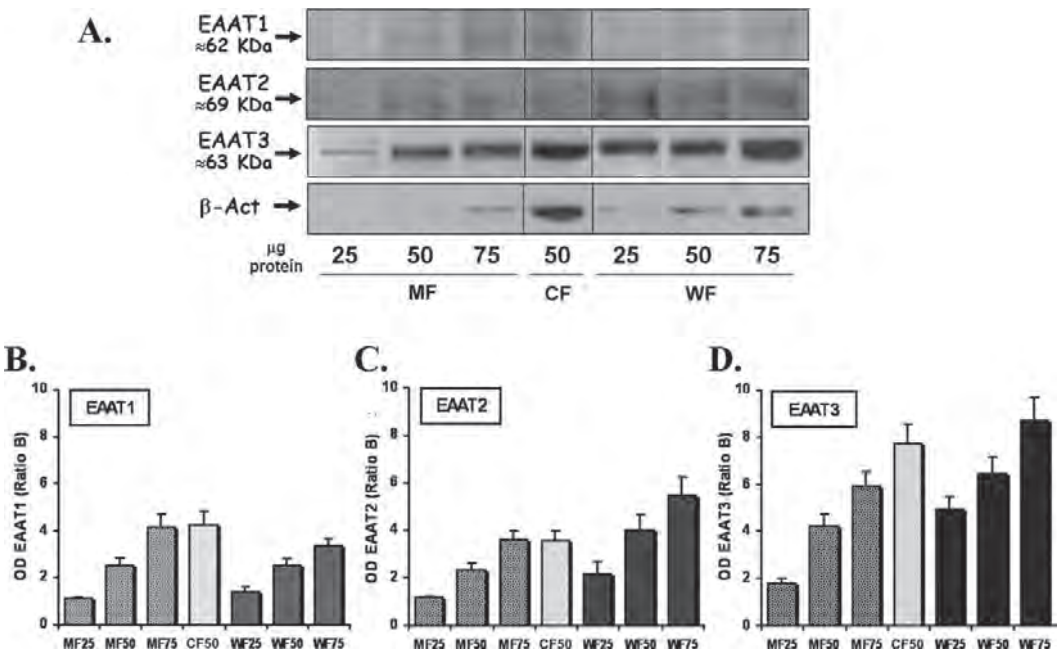

Fig. 6. EAAT1-3 expression in subcellular fractions of bovine chromaffin cells determined by Western blot analysis. **A**: Representative images of Western blot experiments for EAAT1, EAAT2, and EAAT3 in whole fraction (WF), membrane fraction (MF), and cytosolic fraction (CF) of chromaffin cells at indicated amounts of total protein. Estimated molecular weights for each glutamate transporter are indicated. **B–D**: Quantitative analysis of EAAT1 (B), EAAT2 (C), and EAAT3 (D) expressions in indicated subcellular fractions of chromaffin cells. The data represent the ratios of densitometric values normalized against the backgrounds obtained for each Western blot and are mean ± SEM values obtained from three experiments with different cell batches each performed in duplicate. β-Actin signals were obtained in order to assess the purity of different fractions and the quantity of proteins in each assay.

EAAT3, respectively (Fig. 6A). However, EAAT3 expression was significantly higher than that of EAAT2 and EAAT1 ($P < 0.001$; ANOVA test). Moreover, unlike the case with VGLUTs, cytosolic signals for the three EAATs were always higher than those found for membrane fractions (Fig. 6B–D), indicating the existence of some traffic of these transporters to plasma membrane through the cytosol. Band intensities were also directly proportional to the protein amount in the samples.

Chromaffin Cells Express VGLUT1 and VGLUT3 and EAAT3 and EAAT2 mRNAs, Which Show Different Regulation by Cytokines and Glucocorticoids

To delve more deeply into the study of glutamate transporter expression in chromaffin cells and its specific regulation, PCR (semiquantitative for Fig. 7A,B and quantitative for Fig. 7D,E) experiments were set for VGLUT1–3, EAAT1–3, and G3PDH genes. Semiquantitative RT-PCR experiments confirmed the presence of specific mRNAs for VGLUT1 and -3 and not for VGLUT2 (Fig. 7A) or for EAAT1–3 (Fig. 7B). To confirm the validity of the primers used, the same experiments were performed in whole homogenates of rat cerebral cortex (Cx) and hippocampus (Hc). Positive signals were obtained for all tested primers, thus confirming their validity (Fig. 7C). Figure 7D,E presents data on the expression of mRNA for EAAT2 and -3 (Fig. 7D) and for VGLUT1 and -3 (Fig. 7E) mRNA expressions obtained by quantitative real-time PCR techniques (RT-qPCR) after chromaffin cell incubation with TNFα, IFNγ, and LPS and specific combinations of them or dexamethasone. These data show that all the cytokines assayed and LPS were able to increase mRNA EAAT3 and VGLUT1 expression, although these treatments do not significantly affect VGLUT3 or EAAT2 mRNA expressions (Fig. 7D,E). Moreover, treatment with dexamethasone was able to increase mRNA EAAT3 and VGLUT1 expression 2 and 3.5 times, respectively, whereas no statistically significant effects were found in the case of EAAT2 or VGLUT3. These results were confirmed by Western blot experiments (Fig. 8). Thus, the treatment of chromaffin cells with the same cytokines for 24 hr revealed the increase in expression of EAAT3 and VGLUT1 at the protein level, but not those of EAAT2 or VGLUT3, except for some cytokine combinations. In this case, the specific combi-

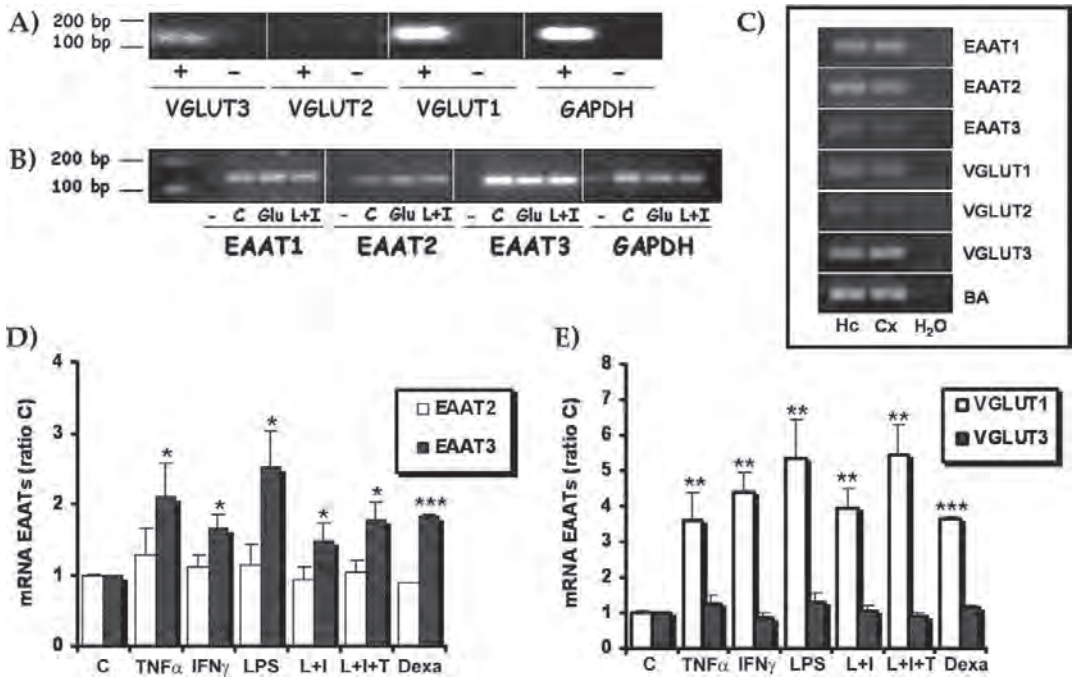

Fig. 7. mRNA expression of different EAATs and VGLUTs in bovine chromaffin cells and regulation of EAATs by cytokines and corticosteroids. **A**: Expression of VGLUTs mRNA in chromaffin cells assayed by conventional RT-PCR. mRNA was extracted with the RNeasy Qiagen Kit and subjected to reverse transcription (RT) and subsequent amplification with an appropriate set of primers for VGLUTs or for GAPDH, used as a housekeeping gene. PCR products RT^- (–) or RT^+ (+) were run on an agarose gel and images collected as described in Materials and Methods. **B**: Regulation of EAAT mRNAs expression in chromaffin cells assayed by conventional RT-PCR. Cells were incubated for 6 hr in the absence (controls; C) or in the presence of 10 μM LPS plus 10 nM IFNγ (L + I) or 1 mM glutamate (Glu). mRNA was extracted and subjected to RT and subsequent amplification with appropriate set of primers for EAATs, and PCR products were run on an agarose gel as described in Materials and Methods. GAPDH was used as a housekeeping gene. **C**: Conventional RT-PCR for mRNA from rat brain cortex (Cx) and hippocampus (Hc) tissues, as positive controls of primers used. **D,E**: For real-time RT-PCR, bovine chromaffin cells were incubated for 6 hr with 10 μM LPS, 10 nM IFNγ, 10 nM TNFα or their specific combinations (L + I or L + I + T) or with 100 nM dexamethasone (Dexa). mRNA was extracted and subjected to reverse transcription and subsequent quantitative amplification with appropriate sets of primers for EAAT2 and -3 (D) or VGLUT1 and -3 (E) and GAPDH as a control, as described in Materials and Methods. Data are expressed as ratios over their controls and are mean ± SEM values obtained from three experiments each performed in triplicate. Statistics compare the effect of cytokines or dexamethasone with their specific controls ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$; one-way ANOVA test).

nations of cytokines potentiate the individual expression separately obtained with each one, an effect that was not apparent at the mRNA expression level, which suggests the existence of additional posttranscriptional or translational regulation. Thus, taken together, these results confirm the expression of specific EAATs and VGLUTs subtypes in chromaffin cells with different regulation by cytokines and glucocorticoids at both mRNA and protein levels.

DISCUSSION

In recent years, evidence has emerged for a role of glutamate as an extracellular signal mediator in the autocrine and/or paracrine system (intestine, testes, placenta, kidney, bone), in addition to its excitatory amino acid neurotransmitter role in the CNS (Skerry and Genever 2001; Hinoi et al., 2004). Recent evidence also points out a role for glutamate as a regulatory molecule in neuroendocrine hypothalamic-hypophysary systems (Hrabovszky and Liposits, 2008) and in endocrine tissues such as pineal gland (Danbolt, 2001) and pancreas where glutamate has a regulatory role in glucagon and insulin secretion (for review see Hinoi et al, 2004). By analogy with neurotransmission in the central glutamatergic synapses, the expression of GLTs could be essential for the termination of glutamate signals by reducing the extracellular concentration in mechanisms underlying possible signal transduction mediated by this amino acid in the adrenal gland. VGLUTs' discovery has provided a reli-

54 Oliván et al.

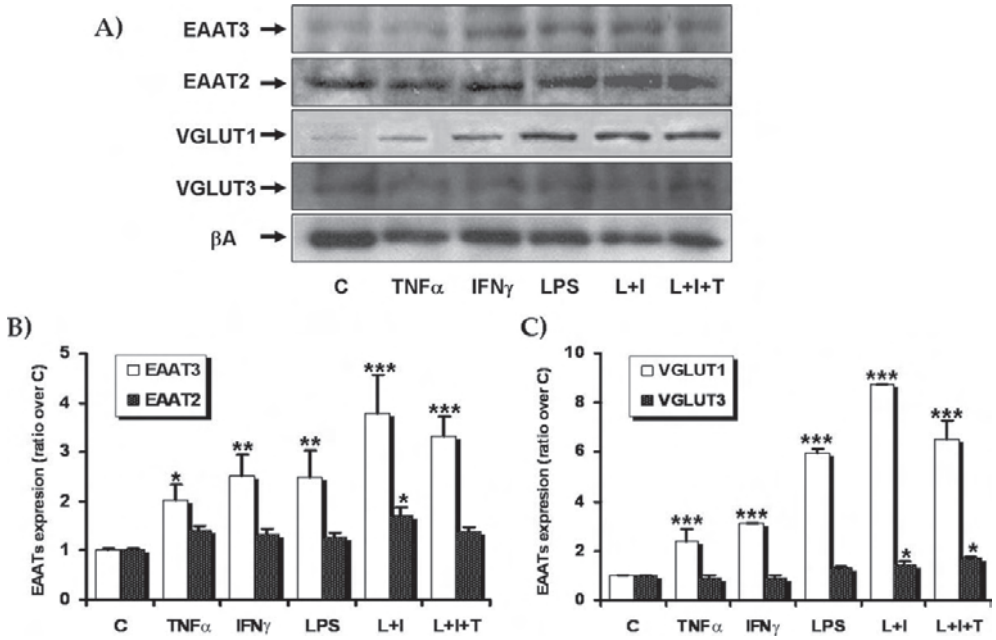

Fig. 8. Regulation of EAATs and VGLUTs expression by cytokines in bovine chromaffin cells, determined by Western blot analysis. Bovine chromaffin cells were incubated for 24 hr with 10 μM LPS, 10 nM IFNγ, 10 nM TNFα, or their specific combinations (L + I or L + I + T), and Western blots were carried out as described in Material and Methods. **A**: Representative images of Western blot experiments for EAAT2, EAAT3, VGLUT1, and VGLUT3 in whole homogenates of chromaffin cells. **B,C**: Quantitative analysis of EAAT2 and -3 (B) or VGLUT1 and -3 (C) expression in chromaffin cells. Data represent the ratios of densitometric values against controls, normalized with β-actin, and are mean ± SEM values obtained from three experiments with different cell batches each performed in duplicate. Statistics compare the effect of cytokines with specific controls for each glutamate transporter (*$P < 0.05$, **$P < 0{,}01$, ***$P < 0.001$; one-way ANOVA test).

able marker of glutamate secretory cells. Because of this, we proposed the study of these transporters in bovine chromaffin cells by several experimental approaches. This paper discusses, in the light of new results on the expression of glutamate transporters in chromaffin cells, the possible role of glutamate in chromaffin cells, which would support the newly proposed nonsynaptic role of glutamate in neuroendocrine communication.

The present study demonstrates, for the first time, that catecholamine-secreting adrenal chromaffin cells exhibit VGLUT1 and -3 and EAAT2 and -3 immunoreactivities and show specific expression of these glutamate transporters at both mRNA and protein levels. The synthesis of VGLUTs and EAATs exhibits robust up-regulation in response to certain endocrine challenges, such as the cytokines TNFα, IFNγ, and LPS, as well as by glucocorticoids such as dexamethasone, indicating that altered glutamatergic signalling may represent an important adaptive mechanism.

Previous results from our group show that ionotropic and metabotropic glutamate receptor agonists can elicit catecholamine secretion and increase intracellular calcium levels in adrenal medulla at endocrine release sites (González et al., 1998). Structural constituents of adrenal medulla, chromaffin cells contain elements of glutamatergic transmission, including glutamate receptors and enzymes of the glutamate/glutamine cycle (Schwendt and Jezová, 2001; Sarriá et al., 2006).

Our previous results also indicate that chromaffin cells release different amounts of glutamate when stimulated by depolarizing agents that act by different mechanisms. In comparison with cortical neurones, the magnitude of glutamate secretion is lower, but it is similar in both the relative percentages of release in any case and the order of potency obtained for different secretagogues in both cell types: KCl > 4-AP > veratridine (Romero et al., 2003). All these catecholamine secretagogues induced glutamate secretion by two mechanisms: 1) a Ca^{2+}-dependent, exocytotic mechanism and 2) a Ca^{2+}-independent mechanism mediated by reversion of the electrogenic glutamate transporter.

Our previous results on the subcellular location of glutamate indicate that 90% of the cell-releasable glutamate pool is located in the cytoplasm and that only 10% is contained in the secretory granules (chromaffin granules and synaptic-like microvesicles; Romero et al., 2003). This glutamate distribution is similar to that of GABA (81% cytosolic and 19% granular; Oset-Gasque

et al., 1990). However, the compartmentation of cellular catecholamines is very different, insofar as 97% of total catecholamines are mainly located in chromaffin granules.

These previous results suggested the presence in chromaffin cells of specific glutamate transporters, whose identity has been revealed in the present work. Thus, by using multitechnical approaches (confocal microscopy, flow cytometric analysis, Western blot, and qRT-PCR techniques), the presence of specific plasma membrane (EAATs) and vesicular glutamate (VGLUTs) transporters has been investigated. We found a specific expression of both EAATs and VGLUTs. With respect to EAATs expression, immunocytochemical flow cytometry analysis showed a very high expression of the neuron-specific transporter EAAT3 (about 95% of cells), followed by EAAT2 (about 65% of cell) and EAAT1 (about 30% of cells). These results were confirmed by Western blot techniques, showing estimated molecular weights of about 69, 63, and 62 kDa for EAAT2, EAAT3, and EAAT1, respectively, which are similar to the weights described for brain (Sánchez-Mendoza et al., 2010) and for dorsal root ganglia and sciatic cells (Carozzi et al., 2008) and by qPCR techniques. However, the low expression obtained for EAAT1 at the protein level, along with the fact that specific expression of EAAT1 was not systematically found in all experiments, urges us to think that this transporter might be more important in the endothelial cells, as it is in astrocytes or endothelial cells at the brain level (Teichberg et al., 2009). The low EAAT1 expression in chromaffin cells could be in agreement with results from Lee et al. (2001) showing that the expression of GLAST (EAAT1) is transiently increased at 3 weeks of age in rat adrenal glands and with the fact that, while GLT1 (EAAT2) is highly expressed throughout the CNS, the expression of GLAST is low and restricted to a few brain regions, such as the Bergmann glia in cerebellum (Nedergaard et al., 2002).

The most important plasmalemmal glutamate transporter found in chromaffin cells is EAAT3, which is what we expected, given the neural origin of these cells. Interestingly, this transporter shows an important regulation by cytokines, such as IFNγ and TNFα, and by LPS and corticosteroids, such as dexamethasone, which is very relevant given the high quantity of these steroid hormones, which come from adrenal cortex, to which these cells are subjected. Thus, this specific regulation of EAAT3, different from that of EAAT2, suggests that the expression of both transporters is not redundant in adrenal medulla, because these transporters could be subjected to different regulation, as demonstrated in the brain. Thgus, it has been shown that, although ischemic preconditioning ''in vivo'' increases the expression of EAAT2 and EAAT3 glutamate transporters, the up-regulation of the latter is, at least partially, mediated by the TNFα-converting enzyme/TNFα/TNFR1 pathway (Pradillo et al., 2006), whereas EAAT2 expression is up-regulated by PPARγ agonists (Romera et al., 2007).

In chromaffin cells, EAATs share their subcellular location between membrane and cytosolic fractions. This is in accordance with previous studies in neurons suggesting that the expression of EAAT3 is located in both neuronal cytoplasm and plasma membrane (Pradillo et al., 2006), although EAAT3 is localized mainly at the plasma membrane level when its up-regulation by ischemic preconditioning takes places (Pradillo et al., 2006). Moreover, a large portion of total EAAT2 (and a minor portion of total EAAT1, EAAT3, and EAAT4) was associated with cholesterol-rich lipid raft microdomains of the plasma membrane, and the association with these cholesterol-rich microdomains is important for its localization and function (Butchbach et al., 2004).

The presence of EAATs in adrenal chromaffin cells seems to support previous data from our group indicating that both neurotransmitters (glutamate and catecholamines) are released to a different extent when chromaffin cells are stimulated by different secretagogues (Romero et al., 2003). The presence of a large pool of cytosolic glutamate in chromaffin cells could explain the greater involvement of EAATs in glutamate release in comparison with catecholamine release. The expression of EAATs along with the mainly cytosolic location of glutamate in chromaffin cells confers upon these glutamate transporters a special relevance in both the glutamate secretory process and the potential excitotoxic effect of glutamate in these cells (Vicente et al., 2006).

Although our previous results demonstrated that vesicular glutamate makes up only 10% of the cell-releasable glutamate pool, this is a large enough pool to account for the measured Ca^{2+}-dependent release (1–7% of total glutamate in 5 min; Romero et al., 2003). Thus, pool size/depletion is not limiting to consider Ca^{2+}-dependent glutamate release as exocytotic secretion. Results presented in this study clearly demonstrate the specific expression of VGLUT1 and -3 in about 55% and 25%, respectively, of the whole population of chromaffin cells. However, we did not find specific expression of VGLUT2, which is also what we expected, given the segregation and complementary expression patterns of both vesicular transporters in glutamatergic neurons from brain (Fremeau et al., 2004). Unlike EAATs, VGLUTs are localized mainly in the membrane fraction. Their estimated molecular weights of about 50 kDa for VGLUT1 and 60 kDa for VGLUT3 are very similar to those found in the brain (Sánchez-Mendoza et al., 2010). RT-qPCR techniques confirm the expression of these glutamate transporters at the mRNA level and show a different regulation between them by cytokines and glucocorticoids. The strong regulation by cytokines and dexamethasone found for VGLUT1 indicates that, as in the case of EAAT3, VGLUT1 could have an important role in the regulation of glutamate release and catecholamine release in the adrenal medulla. In fact, the expression of VGLUT1 mRNA in about 50% of corticotrophs but only in 7.7% of luteinizing hormone gonadotrophs in anterior and intermediate lobes of pituitary has recently been demonstrated (Kocsis

et al., 2010). Thus, the regulation of VGLUT1 in chromaffin cells by glucocorticoids seems to support a putative functional role of this transporter in the response to glutamate in chromaffin cells. The fact that EAAT3 and VGLUT1 undergo a similar regulation by cytokines and glucocorticoids suggests the existence of a functional relationship between the two transporters in control of extracellular glutamate levels in the adrenal medulla. Our recent data showing the up-regulation of nNOS by glucocorticoids and iNOS by cytokines in chromaffin cells (Pérez-Rodríguez et al., 2009), together with the fact that NO increases catecholamine (Oset-Gasque et al., 1994) and glutamate secretion (unpublished results), seem to support this conclusion. Moreover, these results support previous evidence showing the presence of VGLUT1 [known first as differentiation-associated Na^+-dependent inorganic phosphate cotransporter (DNPI), as an isoform of brain specific BNPI], in neuroendocrine cells, such as α cells in Langerhans islets and pynealocytes (Hayashi et al., 2001), adding important further evidence for a role of L-glutamate as an intracellular signaling molecule in endocrine peripheral organs with an important role in the regulation of hormone secretion. In addition, glutamate in adrenal medulla could serve as a precursor for GABA synthesis.

Therefore, taken together, the interesting results presented here, together with our previous results on glutamate release from adrenal medulla (Romero et al., 2003) and its role in apoptosis induced by NO in chromaffin cells (Pérez-Rodríguez et al., 2009), support 1) the participation of these glutamate transporters in the process of glutamate transport and release in chromaffin cells and in the regulation of their neurosecretory function in adrenal medulla and 2) the validity of chromaffin cells as a model for the study of neurosecretion, neurotransmission, and neurodegeneration mechanisms.

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